ECOLOGICAL PHYSIOLOGY OF DESERT WOODRATS (*NEOTOMA LEPIDA*) WITH RESPECT TO AMBIENT TEMPERATURE AND DIETARY TOXINS

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Intrinsic factors like nutrients and plant toxins are well known to influence the feeding behavior of mammalian herbivores. However, far less is known about the impact of extrinsic factors such as ambient temperature on herbivore diet selection. Growing evidence suggests that plant secondary compounds become more toxic at higher temperatures due to decreased liver function. This phenomenon, known as temperature-dependent toxicity (TDT), could have critical implications for mammalian herbivores that must balance homeothermy with xenobiotic metabolism in a warming environment. Very little study of TDT has been done in natural systems. For my dissertation, I investigated TDT in a small herbivorous rodent, the desert woodrat (*Neotoma lepida*). In the Mojave Desert, this species feeds on creosote bush (*Larrea tridentata*), which produces numerous toxic plant secondary compounds. I explored aspects of TDT with a multilevel investigation using physiological assays, molecular analyses, and behavioral observations. First, I determined that warmer ambient temperatures reduced overall liver function in desert woodrats. Second, I investigated a potential underlying mechanism of TDT by studying the impact of temperature and plant toxins on differential gene expression in the liver of desert woodrats. More genes were up-regulated at cooler temperatures, suggesting that lower temperatures offer a "release state", permitting induction of numerous genes in the liver. To explore the impacts of TDT on woodrats, I

next tested how temperature affected tolerance to ecologically relevant plant toxins (i.e., creosote resin). Increased temperatures limited the ingestion of creosote resin in desert woodrats and reduced their ability to maintain body mass on a controlled dose of creosote resin. Finally, I examined whether woodrats could behaviorally mitigate the negative effects of TDT by utilizing cooler microclimates. Woodrats not only used cooler microclimates when provided access, but also displayed less body mass loss compared to woodrats without microclimate access. The interactions of ambient temperature and plant toxins present a novel dimension to investigations on herbivore foraging, and further study of TDT will advance the field of plant-animal interactions. Together, my work has expanded our understanding of the challenges that mammalian herbivores may face as global temperatures rise.

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CHAPTER 1

INTRODUCTION

Mammalian herbivores face challenges at every meal. Plants are a nutrient-poor food source, low in nitrogen and high in indigestible fiber. As a result, many herbivorous species need to ingest large amounts of plant material each day to meet energy needs (Dearing et al. 2005a). Additionally, plants produce secondary compounds as a defense against herbivory that can occur in high concentrations and be chemically diverse (Freeland and Janzen 1974). Intrinsic factors such as nutrients and toxins have long governed theories of herbivore diet selection, yet extrinsic factors such as ambient temperature have not traditionally been considered. However, there is growing evidence that there is a strong interaction between toxic compounds and ambient temperature, and this interaction could gravely impact mammalian herbivores (Dearing 2013). This phenomenon is known as temperature-dependent toxicity (TDT) and, in short it predicts that compound toxicity increases with ambient temperature.

Evidence for TDT can be found across a range of scientific fields. Work from pharmacology has documented that warmer temperatures resulted in lower lethal doses of toxic chemicals and longer drug clearance times of the liver in laboratory rats (Keplinger et al. 1959, Kaplanski and Ben-Zvi 1980). Rats also chose cooler ambient temperatures immediately following an acute toxic challenge when provided a thermal gradient

(Gordon and Stead 1986a, b). In addition, studies from agricultural science have found that higher temperatures increased the severity of fescue toxicosis in cattle (Aldrich et al. 1992, Sales et al. 2011). Fescue toxicosis is a suite of deleterious symptoms caused by toxins produced by fungal endophytes, which infect tall fescue grasses (Spiers et al. 2008). Experiments with laboratory rats also provided evidence for a shift in hepatic gene expression whereby xenobiotic metabolism was down-regulated at warmer temperatures (Settivari et al. 2009). Yet, there is limited evidence for TDT in a natural system with a herbivore feeding on an ecologically and evolutionarily relevant diet of plant secondary compounds. TDT could be a major challenge for mammalian herbivores that encounter plant toxins at every meal and may likely face warmer temperature regimes as the climate changes.

This thesis investigated TDT within a small, herbivorous rodent, the desert woodrat (*Neotoma lepida*). I worked with a population of *N. lepida* that inhabit the Mojave Desert and ingest large amounts of creosote bush (*Larrea tridentata*), whose leaves are coated with resin composed of hundreds of different compounds known to cause kidney cysts and liver damage in laboratory rodents (Cameron and Rainey 1972, Meyer and Karasov 1989, Rios et al. 2008). Arid environments are currently experiencing more extreme impacts of climate change such as heat waves and droughts than temperate or tropical environments (Jardine et al. 2013, Field et al. 2014); therefore, rising ambient temperatures are a real and imminent threat to the desert woodrat.

Reduced liver function at higher ambient temperatures is thought to be the cause of TDT. As the largest visceral organ, the liver is responsible for detoxification of foreign compounds and also produces heat as a by-product of enzymatic reactions. Mammals are

faced with a unique challenge to balance the requirements of thermoregulation with xenobiotic metabolism. Although the hypothesis of reduced liver function has been tested in pharmacological studies with laboratory rodents, I was the first to test the effects of warmer temperatures in an ecologically and evolutionarily relevant system. In Chapter 2, I utilized a whole-organism measure of liver function and found that higher ambient temperatures decreased liver function in the desert woodrat.

In Chapter 3, I used microarrays custom-designed for *N. lepida* to determine changes in gene expression, adding a molecular-level analysis to the investigation of TDT. The underlying cause of decreased liver function at warmer temperatures is predicted to be reductions in hepatic gene expression. My results imply that ambient temperature influenced gene expression profiles in the desert woodrat to a greater extent than dietary plant toxins. Specifically, a greater number and diversity of genes were differentially expressed at 22°C compared to 27°C, suggesting that cooler temperatures might be a "release state" for the liver.

Reduced liver function could have detrimental implications for mammalian herbivores that consume plant toxins at every meal. In Chapter 4, I tested the effect of ambient temperature on the desert woodrats' tolerance to dietary creosote resin. These experiments provided evidence that higher temperatures resulted in a reduced tolerance to creosote resin compared to cooler temperatures. Tolerance was measured by the maximum dose of creosote resin willingly ingested by woodrats and by the ability of woodrats to maintain body mass while eating a controlled dose of resin.

Lastly, I was interested in whether herbivores could behaviorally mitigate the negative effects of TDT by utilizing cooler microclimates when challenged by warmer

temperatures and a plant toxin-rich diet. In Chapter 5, I quantified microclimate use by woodrats in cages that provided access to different temperatures. I also collected temperatures from woodrat nests in the Mojave Desert from May 2014 – October 2015. I found that dietary toxins did not influence cooler microclimate use by desert woodrats. Food intake did not change with microclimate access, but woodrats lacking access to cooler temperatures lost more body mass than animals with access. Additionally, temperature profiles from active nests suggest that some middens provide woodrats with cooler microclimates in their natural habitat. My results provide preliminary evidence that cooler refugia could reduce the effects of TDT on the desert woodrat.

Taken together, the results of this dissertation elucidate a previously unrecognized challenge to mammalian herbivores. After only a 5-8°C increase in ambient temperature, I found that clearance times of a proxy compound were longer, fewer genes were differentially expressed in the liver, and desert woodrats experienced a decrease in their ability to ingest dietary toxins and thus, their main food source, creosote bush. The use of cooler microclimates, which exist in their natural habitat, could offer a way for desert woodrats to combat these negative effects of TDT. However, if cooler refugia disappear from the thermal landscape, woodrats may be required to drastically change their behavior. One approach would be to alter their foraging strategy to incorporate less toxic food sources. Yet more time spent searching for food could lead to greater predation risks. Alternatively, woodrats may move to a new habitat with different temperature regimes and food sources. Furthermore, the results presented here could be expanded beyond woodrats to other mammals and potentially other endotherms, which are faced with the same task of balancing thermoregulation with detoxification. Going forward, the

interactions between intrinsic plant toxins and extrinsic temperature are too important to be ignored in models of herbivore diet selection and plant-animal interactions.

In the context of climate change, TDT could be acting in concert with other stressors already impacting mammalian herbivores. Range shifts have already been documented in small mammalian species, including *Neotoma*, to higher elevations and latitudes (Moritz et al. 2008, Rowe et al. 2011). It is possible that mammalian herbivores have been able to cope with TDT in the past but now face historically unprecedented temperatures and extreme weather events. Understanding the physiological mechanisms that impact behavioral changes in mammalian herbivores will be critical for future management of these important components in numerous ecosystems.

References

Aldrich, C. G., J. A. Paterson, J. L. Tate, and M. S. Kerley. 1992. The effects of endophyte-infected tall fescue consumption on diet utilization and thermal regulation in cattle. Journal of Animal Science 71:164-170.

Cameron, G. N. and D. G. Rainey. 1972. Habitat utilization by Neotoma lepida in the Mohave desert. Journal of Mammalogy 53:251-266.

Dearing, M. D. 2013. Temperature-dependent toxicity in mammals with implications for herbivores: a review. Journal of Comparative Physiology B 183:43-50.

Dearing, M. D., W. J. Foley, and S. McLean. 2005. The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. Annual Review of Ecology, Evolution, and Systematics:169-189.

Field, C. B., V.R. Barros, D.J. Dokken, K.J. Mach, M.D. Mastrandrea,, M. C. T.E. Bilir, K.L. Ebi, Y.O. Estrada, R.C. Genova, B. Girma, E.S. Kissel, A.N. Levy, S. MacCracken,, and a. L. L. W. e. P.R. Mastrandrea, editors. 2014. Climate Change 2014: Impacts, Adapation, and Vulnerability. Cambridge University Press, New York, NY.

Freeland, W. J. and D. H. Janzen. 1974. Strategies in Herbivory by Mammals: The Role of Plant Secondary Compounds. American Naturalist 108:269-289.

Gordon, C. J. and A. G. Stead. 1986a. Effect of alcohol on behavioral and autonomic

thermoregulation in mice. Alcohol 3:339-343.

Gordon, C. J. and A. G. Stead. 1986b. Effect of nickel and cadmium chloride on autonomic and behavioral thermoregulation in mice. Neurotoxicology 7:97-106.

Jardine, A., R. Merideth, M. Black, and S. LeRoy. 2013. Assessment of climate change in the southwest United States: a report prepared for the National Climate Assessment. Island press.

Kaplanski, J. and Z. Ben-Zvi. 1980. Effect of chronic heat exposure on in-vitro drug metabolism in the rat. Life Sciences 26:639-642.

Keplinger, M. L., G. E. Lanier, and W. B. Deichmann. 1959. Effects of environmental temperature on the acute toxicity of a number of compounds in rats. Toxicology 1:156-161.

Meyer, M. W. and W. H. Karasov. 1989. Antiherbivore chemistry of Larrea tridentata: effects on woodrat (*Neotoma lepida*) feeding and nutrition. Ecology:953-961.

Moritz, C., J. Patton, C. Conroy, J. Parra, G. White, and S. Beissinger. 2008. Impact of a century of climate change on small-mammal communities in Yosemite National Park, USA. Science 322:261-264.

Rios, J. M., A. M. Mangione, and J. C. Gianello. 2008. Effects of natural phenolic compounds from a desert dominant shrub Larrea divaricata Cav. on toxicity and survival in mice. Revista Chilena de Historia Natural 81:293-302.

Rowe, R., R. Terry, and E. Rickart. 2011. Environmental change and declining resource availability for small-mammal communities in the Great Basin. Ecology 92:1366-1375.

Sales, M. A., K. Y. Murphy, S. T. Reiter, A. H. Brown, Jr., M. A. Brown, M. L. Looper, and C. F. Rosenkrans, Jr. 2011. Effects of forage type, body condition and single-nucleotide polymorphisms in the bovine cytochrome P450 regulatory region on cow productivity. Journal of Animal Physiolgy and Animal Nutrition 97:91-96.

Settivari, R., T. Evans, L. Yarru, P. Eichen, P. Sutovsky, G. Rottinghaus, E. Antoniou, and D. Spiers. 2009. Effects of short-term heat stress on endophytic ergot alkaloid-induced alterations in rat hepatic gene expression. Journal of Animal science 87:3142-3155.

Spiers, D. E., T. J. Evans, and G. E. Rottinghaus. 2008. Interaction between thermal stress and fescue toxicosis: Animal models and new perspectives.*in* C. P. W. C.A. Roberts, D.E. Spiers, editor. Neotyphodium in Cool-Season Grasses. Blackwell Publishing Ltd, Oxford, UK.

CHAPTER 2

WARMER AMBIENT TEMPERATURES DEPRESS LIVER FUNCTION IN A MAMMALIAN HERBIVORE

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Research

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Physiology

Warmer ambient temperatures depress liver function in a mammalian herbivore

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Diet selection in mammalian herbivores is thought to be mainly influenced by intrinsic factors such as nutrients and plant secondary compounds, yet extrinsic factors like ambient temperature may also play a role. In particular, warmer ambient temperatures could enhance the toxicity of plant defence compounds through decreased liver metabolism of herbivores. Temperature-dependent toxicity has been documented in pharmacology and agriculture science but not in wild mammalian herbivores. Here, we investigated how ambient temperature affects liver metabolism in the desert woodrat, *Neotoma lepida*. Woodrats ($n = 21$) were acclimated for 30 days to two ambient temperatures (cool = 21°C, warm = 29°C). In a second experiment, the temperature exposure was reduced to 3.5 h. After temperature treatments, animals were given a hypnotic agent and clearance time of the agent was estimated from the duration of the hypnotic state. The average clearance time of the agent in the long acclimation experiment was 45% longer for animals acclimated to 29°C compared with 21°C. Similarly, after the short exposure experiment, woodrats at 29°C had clearance times 26% longer compared with 21°C. Our results are consistent with the hypothesis that liver function is reduced at warmer environmental temperatures and may provide a physiological mechanism through which climate change affects herbivorous mammals.

1. Introduction

Diet selection in mammalian herbivores is well known to be influenced by intrinsic factors such as nutrients and plant secondary compounds as well as by their interactions [1]. However, the influence of environmental factors like ambient temperature has received less attention. There has been growing evidence for a phenomenon called temperature-dependent toxicity (TDT) [2]. Under this paradigm, the toxicity of dietary plant secondary compounds is predicted to increase at warmer temperatures owing to interactions between ambient temperature and mammalian physiology. If plant secondary compounds are more toxic to mammalian herbivores at higher temperatures, then a warming environment could greatly affect the diet selection of mammalian herbivores.

Multiple lines of evidence provide support for TDT. Pharmacologists have reported decreases in the lethal dose of numerous plant-derived compounds when administered to laboratory rodents at higher ambient temperatures [3]. Agricultural scientists have documented that a deleterious condition in cattle known as fescue toxicosis, caused by fungal toxins in infected grasses, was exacerbated at warmer temperatures [4]. Lastly, ecologists have shown that herbivorous rodents experienced temperature-mediated changes in the selection of diets containing plant secondary compounds [5].

TDT is thought to be caused by a decrease in liver function at warmer temperatures. The liver is responsible for drug metabolism, and as the largest visceral organ, also plays a key role in thermoregulation [6]. Previous work in laboratory rodents has documented decreases in hepatic gene expression of xenobiotic metabolism and in hepatic enzyme activity at warmer temperatures [7,8]. Energetic processes like biotransformation may become compromised in mammals at higher temperatures as they reach their maximal capacity to dissipate body heat [9].

Table 1. Body mass (mean ± s.e.) of desert woodrats (*N. lepida*) before and after two temperature experiments. Sample size for each experiment is indicated.

	long, 30 day acclimation ($n = 13$)		short, 3.5 h exposure ($n = 15$)
temperature	before (g)	after (g)	after (g)
cool	113.6 (±6.9)	119.3 (±7.3)	115.8 (±3.1)
warm	120.1 (±7.8)	123.7 (±8.2)	115.6 (±3.1)

The hypothesis that liver function decreases with increasing ambient temperature has not been tested in an ecologically and evolutionarily relevant system. Here, we compared liver function of herbivorous rodents exposed to two ambient temperatures with a non-destructive assay. Two experiments were conducted whereby animals were acclimated to temperature treatments for 30 days or exposed for 3.5 h prior to measuring liver function. We predicted that liver function of herbivorous rodents would decrease at warmer temperatures.

2. Material and methods

Woodrats (genus *Neotoma*) are nocturnal, herbivorous rodents that feed on an array of toxic plants [10,11]. Populations of the desert woodrat (*Neotoma lepida*) inhabiting the Mojave Desert feed mainly on creosote bush (*Larrea tridentata*), the resin of which contains toxic phenolic compounds [12,13]. We collected 21 animals from the Mojave Desert near Beaver Dam, Utah (37°06′ N, 113°58′ W) in October 2010 and July 2011. Animals were brought to the University of Utah (Salt Lake City), maintained at room temperature (23–25°C) on a 12 L : 12 D cycle and fed rabbit chow (Harlan Teklad 2031) for at least three months before experiments.

The two temperature treatments were 'cool' (21°C) and 'warm' (29°C). As in our previous studies [5,14], these temperatures were selected to be within the thermal neutral zone (29°C) or just below this zone (21°C) for *N. lepida* (see electronic supplementary material, figure S1). The temperatures are commonly experienced by animals in the Mojave Desert for up to six months of the year based on monthly mean and daily maximum values (see the electronic supplementary materials). All animals were exposed to cool and warm temperature treatments after which non-destructive liver function assays were conducted. A cross-over design was used to control for temperature order. Two experiments were conducted with different acclimation periods. A longer, 30 day acclimation period permitted physiological changes to occur, similar to responses across seasons (e.g. changes in insulation). A shorter, 3.5 h exposure period removed any acclimation effect and better reflected daily temperature fluctuations in nature. Animals were returned to room temperature for 3 days between temperature treatments during the 30 day experiment and for at least two weeks during the 3.5 h experiment. Room temperatures were controlled with thermostats and space heaters (Delonghi Safe Heat, Model DUH30; electronic supplementary material, table S1).

Hypnotic state assays were conducted to measure liver function after each temperature treatment. These assays are a validated method of whole-organism liver function and are non-destructive, which allow paired *t*-test analyses [8]. Briefly, a hypnotic agent was administered (IP, 100 mg kg^{-1}) 4 h into the light cycle, and animals were returned to home cages. The length of time spent in the hypnotic state, as indicated by the inability of the animal to right itself, was recorded for every animal in each temperature treatment as a proxy for toxin clearance time. Longer clearance times indicated decreased liver function. The hypnotic agent, hexobarbital, was used as a proxy compound for dietary toxins consumed by this species [15,16]. A minimum wash-out period of seven days was observed after each assay. No difference in clearance time between light and dark cycles was expected because toxin concentration has a greater influence on hepatic systems involved with drug metabolism compared with circadian rhythmicity in rodents [17]. Moreover, after the hexobarbital injection, animals were placed on their back, which is not a natural sleeping posture for woodrats.

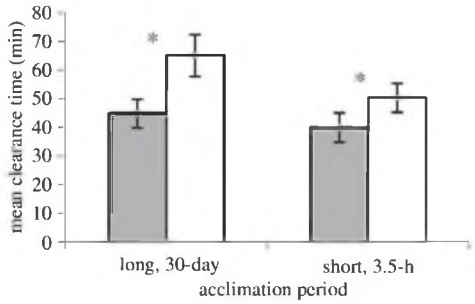

Figure 1. Clearance time (mean ± s.e.) of hexobarbital in desert woodrats (*N. lepida*) at two ambient temperatures (grey = 21°C, white = 29°C) during two acclimation periods. Asterisks indicate $p < 0.05$.

3. Results

Liver function of the desert woodrat was significantly lower at higher ambient temperatures. After the 30 day experiment, average clearance times were 45% longer in the warm temperature group compared with the cool group ($t = -3.31$; d.f. = 12; $p = 0.006$; figure 1). After the 3.5 h experiment, average clearance times were 26% longer in the warm group compared with the cool group ($t = -2.15$; d.f. = 14; $p = 0.049$; figure 1).

We observed qualitative changes in nesting behaviour during the 30 day experiment. Cool-acclimated animals constructed large nests out of paper towels, shavings and cotton batting, and were often found in their nests (figure 2*a*). By contrast, warm-acclimated animals cleared all bedding material from an area of the home cage (figure 2*b*) and were often found sprawled in the cleared area.

Body mass remained relatively unchanged during experiments (table 1). There was no difference in average body mass between cool and warm temperature groups prior to the long acclimation period ($t = -1.67$; d.f. = 12; $p = 0.12$), however, the temperature groups differed slightly after the long acclimation period ($t = -2.17$; d.f. = 12; $p = 0.051$). Average body mass did not differ between temperature groups after the short exposure period ($t = 0.13$; d.f. = 14; $p = 0.898$).

4. Discussion

Evidence from pharmacology and agricultural science has demonstrated that toxicity increases with elevated temperatures,

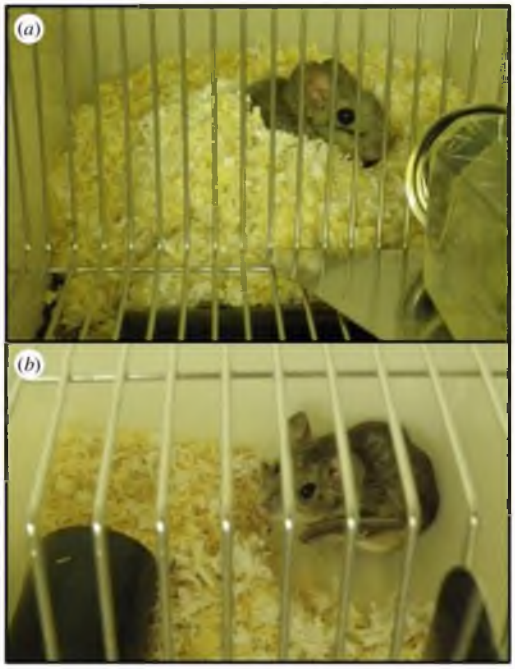

Figure 2. *Neotoma lepida* nesting behaviour at (*a*) cool, 21°C and (*b*) warm, 29°C temperatures. (Online version in colour.)

yet this phenomenon has remained untested in an ecologically and evolutionarily relevant system. Here, we investigated the effect of ambient temperature on mammalian liver function in the desert woodrat. Our results revealed that liver clearance is dependent on ambient temperature, and animals at warmer temperatures showed significantly reduced clearance compared with cooler temperatures. This pattern is apparent regardless of temperature exposure time (figure 1), representing the lack of an acclimation effect. It is worth noting that the warm temperature (29°C) was within *N. lepida*'s thermal neutral zone, which is the range of ambient temperatures were metabolic rates are lowest and considered optimal with respect to energy use. Our data suggest that even acute exposures to warmer temperatures could significantly impair liver function and potentially the detoxification capacity of the desert woodrat. With up to 75% of its diet comprised creosote bush [13], *N. lepida* ingest remarkable doses of toxins at every meal and increased toxicity caused by a warming environment could become an insurmountable challenge.

The physiological mechanism responsible for reduced detoxification capacity in the mammalian liver with increasing temperature is speculative. Laboratory rodents experienced decreases in both hepatic enzyme activity and gene expression of key biotransformation enzymes at warmer temperatures [7,8]. The underlying cause of these decreases is most probably the result of an increase in peripheral circulation under high temperatures to increase heat dissipation and protect the liver [18].

It is possible that small mammals like woodrats may be able to mitigate the reduction in liver function by seeking out cooler microclimates. The desert environment is thermally heterogeneous and cooler ambient temperatures may be present in places like drainages. As detoxification occurs on the scale of minutes to hours, animals using such microclimates may be more exposed to predation during this time. Regardless, the use of behavioural adaptations by herbivores for mitigation remains to be examined.

Although this study investigated a single species, our results may have critical implications for other mammalian herbivores given predicted temperature regimes. As warmer temperatures result in decreased liver function, mammalian herbivores may be forced to change their foraging strategy to reduce intake of toxic plant secondary compounds [2]. However, alternative food sources may be unavailable. If herbivores are forced to decrease toxin intake without an alternative food source, then these mammals would probably experience a reduction in overall energy intake. In a potentially cascading effect, herbivores might move to other environments. Thus, through decreased liver function, TDT may be able to partially explain the observed population shifts of small mammalian herbivores to higher elevations and latitudes [19,20]. In fact, a recent review found food availability to be the most common cause for climate-mediated extinctions [21]. Our results suggest that predictions of increased surface temperature and extreme weather events like heat-waves [22] may pose a yet unrecognized threat to mammalian herbivores, which comprise over 40% of extant mammalian species [23].

Hypnotic state assays offer multiple benefits as a measurement of liver function. Unlike traditional assays that require liver tissue and specialized equipment, hypnotic state assays are non-destructive thereby permitting repeated measurements from a single individual. Additionally, the hepatic enzymes that metabolize hypnotic agents also metabolize plant toxins. Hexobarbital, the agent used in this study, is metabolized by cytochromes P450 2B that are important for the detoxification of plant toxins found in creosote bush [15,16]. Lastly, hypnotic state assays are a tool accessible to physiological ecologists working with non-model species that can aid in elucidating often complex plant–mammal interactions [15]. Thus, further study of TDT in a wide range of mammalian herbivores is achievable.

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References

1. Freeland WJ, Janzen DH. 1974 Strategies in herbivory by mammals: the role of plant secondary compounds. *Am. Nat.* **108**, 269–289. (doi:10.2307/2459891)
2. Dearing MD. 2013 Temperature-dependent toxicity in mammals with implications for herbivores: a review. *J. Comp. Physiol. B* **183**, 43–50. (doi:10.1007/s00360-012-0670-y)
3. Keplinger ML, Lanier GE, Deichmann WB. 1959 Effects of environmental temperature on the acute toxicity of a number of compounds in rats. *Toxicology* **1**, 156–161.

4. Spiers DE, Evans TJ, Rottinghaus GE. 2008 Interaction between thermal stress and fescue toxicosis: animal models and new perspectives. In *Neotyphodium in cool-season grasses* (eds CA Roberts, CP West, DE Spiers), pp. 243–270. Oxford, UK: Blackwell Publishing Ltd.
5. Dearing MD, Forbey JS, McLister JD, Santos L. 2008 Ambient temperature influences diet selection and physiology of an herbivorous mammal, *Neotoma albigula*. *Physiol. Biochem. Zool.* **81**, 891–897. (doi:10.1086/588490)
6. Baconnier P, Benchetrit G, Tanche M. 1979 Liver heat production and temperature regulation in the anesthetized dog. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **6**, R334–R339.
7. Settivari R, Evans T, Yarru L, Eichen P, Sutovsky P, Rottinghaus G, Antoniou E, Spiers D. 2009 Effects of short-term heat stress on endophytic ergot alkaloid-induced alterations in rat hepatic gene expression. *J. Anim. Sci.* **87**, 3142–3155. (doi:10.2527/jas.2008-1684)
8. Kaplanski J, Ben-Zvi Z. 1980 Effect of chronic heat exposure on in-vitro drug metabolism in the rat. *Life Sci.* **26**, 639–642. (doi:10.1016/0024-3205(80)90240-4)
9. Speakman J, Król E. 2010 Maximal heat dissipation capacity and hyperthermia risk: neglected key factors in the ecology of endotherms. *J. Anim. Ecol.* **79**, 726–746. (doi:10.1111/j.1365-2656.2010.01689.x)
10. Karasov WH. 1989 Nutritional bottleneck in a herbivore, the desert wood rat (*Neotoma lepida*). *Physiol. Zool.* **62**, 1351–1382.
11. Vaughan TA. 1982 Stephens' woodrat, a dietary specialist. *J. Mammal.* **63**, 53–62. (doi:10.2307/1380671)
12. Meyer MW, Karasov WH. 1989 Antiherbivore chemistry of *Larrea tridentata*: effects on woodrat (*Neotoma lepida*) feeding and nutrition. *Ecology* **70**, 953–961. (doi:10.2307/1941362)
13. Cameron GN, Rainey DG. 1972 Habitat utilization by *Neotoma lepida* in the Mohave desert. *J. Mammal.* **53**, 251–266. (doi:10.2307/1379160)
14. McLister J, Sorensen J, Dearing M. 2004 Effects of consumption of juniper (*Juniperus monosperma*) on cost of thermoregulation in the woodrats *Neotoma albigula* and *Neotoma stephensi* at different acclimation temperatures. *Physiol. Biochem. Zool.* **77**, 305–312. (doi:10.1086/380211)
15. Dearing MD, Skopec MM, Bastiani MJ. 2006 Detoxification rates of wild herbivorous woodrats (*Neotoma*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **145**, 419–422. (doi:10.1016/j.cbpa.2006.07.016)
16. Malenke JR, Magnanou E, Thomas K, Dearing MD. 2012 Cytochrome P450 2B diversity and dietary novelty in the herbivorous, desert woodrat (*Neotoma lepida*). *PLoS ONE* **7**, e41510. (doi:10.1371/journal.pone.0041510)
17. Davidson AJ, Castanon-Cervantes O, Stephan FK. 2004 Daily oscillations in liver function: diurnal vs circadian rhythmicity. *Liver Int.* **24**, 179–186. (doi:10.1111/j.1478-3231.2004.0917.x)
18. Hales J, Rowell L, King R. 1979 Regional distribution of blood flow in awake heat-stressed baboons. *Am. J. Physiol. Heart Circ. Physiol.* **237**, H705–H712.
19. Rowe R, Terry R, Rickart E. 2011 Environmental change and declining resource availability for small-mammal communities in the Great Basin. *Ecology* **92**, 1366–1375. (doi:10.1890/10-1634.1)
20. Moritz C, Patton J, Conroy C, Parra J, White G, Beissinger S. 2008 Impact of a century of climate change on small-mammal communities in Yosemite National Park, USA. *Science* **322**, 261–264. (doi:10.1126/science.1163428)
21. Cahill A *et al.* 2013 How does climate change cause extinction? *Proc. R. Soc. B* **280**, 20121890. (doi:10.1098/rspb.2012.1890).
22. Parry ML, Canziani OF, Palutikof JP, Van der Linden PJ, Hanson CE. 2007 Climate change 2007: impacts, adaptation and vulnerability. Contribution of working group II to the fourth assessment report of the intergovernmental panel on climate change, p. 982. Cambridge, UK: Cambridge University Press.
23. Price SA, Hopkins SSB, Smith KK, Roth VL. 2012 Tempo of trophic evolution and its impact on mammalian diversification. *Proc. Natl Acad. Sci. USA* **109**, 7008–7012. (doi:10.1073/pnas.1117133109).

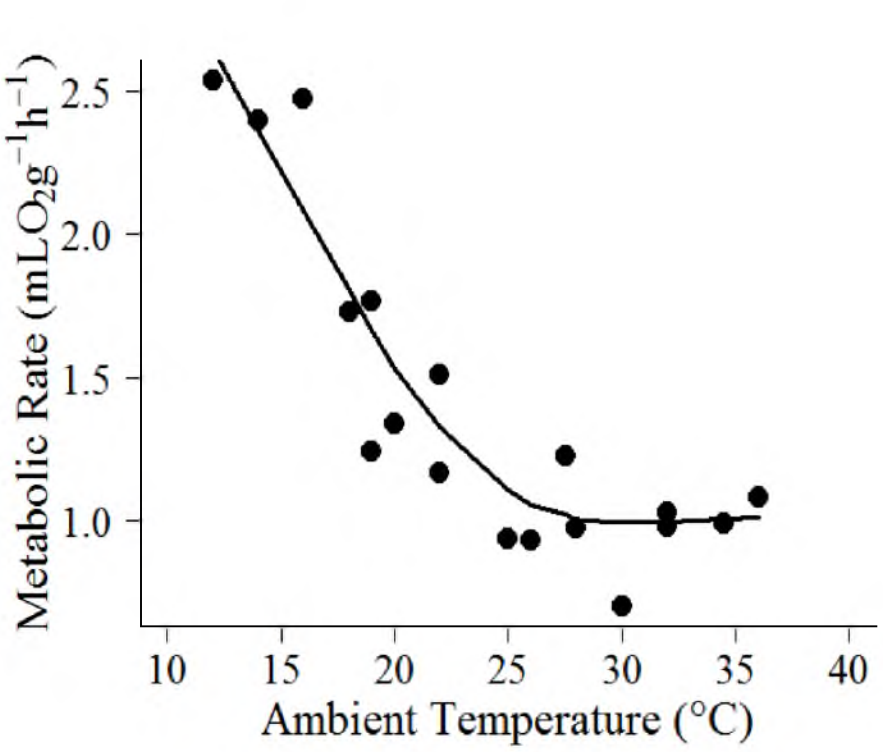

Figure 2.S1. Relationship between metabolic rate and ambient temperature of three desert woodrats (*Neotoma lepida)*. The thermal neutral zone extends from approximately 25-35°C, as indicated by the flat black line. Line made with lowess smoothing function in R (www.r-project.org).

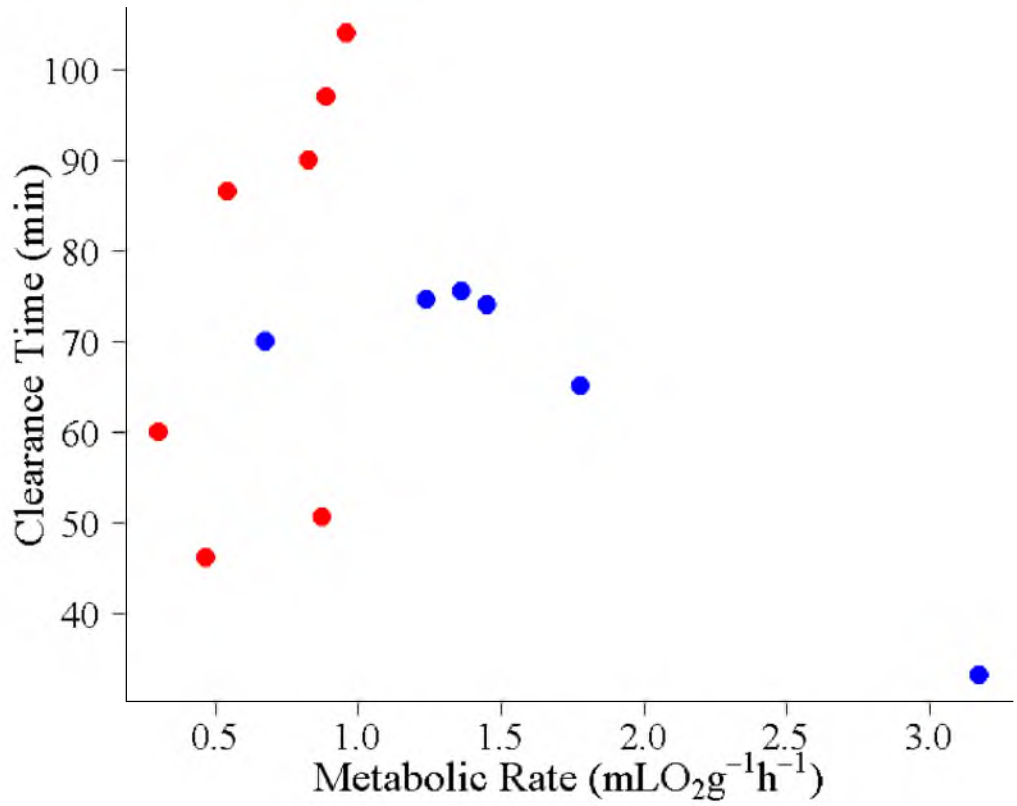

Figure 2.S2. Preliminary relationship between metabolic rates and clearance times after a long, 30-day acclimation period at two ambient temperatures (blue = 21°C, red = 29°C) in *Neotoma lepida*. Each point represents a single individual. Clearance times are indicative of liver function; longer times equate to reduced liver function.

Table 2.S1. Ambient temperature (mean ± 1 s.e.) of animal rooms (cool or warm) after two acclimation periods.

	long, 30-day acclimation		short, 3.5-hour exposure	
	cool	warm	cool	warm
maximum (°C)	22.8 ± 0.2	29.4 ± 0.2	20.6 ± 0.9	28.9 ± 0.2
minimum (°C)	21.1 ± 0.2	27.8 ± 0.2	20.0 ± 0.5	28.3 ± 0.3

CHAPTER 3

TEMPERATURE-MEDIATED CHANGES IN HEPATIC GENE EXPRESSION OF MAMMALIAN HERBIVORES

Abstract

At every meal, herbivores encounter plant toxins produced as a defense against herbivory. Recent work suggests that the toxicity of poisonous compounds is exacerbated by rising ambient temperatures. This phenomenon, known as temperature-dependent toxicity (TDT), is the likely result of decreased liver function at warmer temperatures; however, the underlying cause of TDT remains unknown. In the present study, the effects of temperature and dietary plant toxins on differential gene expression in the liver were compared in herbivorous rodents (woodrats, genus *Neotoma*) using microarrays custom-designed for woodrats. Expression profiles revealed a greater number of differentially expressed genes at 22°C compared to a thermonetural temperature (27°C). Genes and pathways up-regulated at 22°C were related to growth and biosynthesis, suggesting the liver could be in a building state at this temperature. Conversely, genes and pathways up-regulated at 27°C were associated with apoptosis, gluconeogenesis, and protein misfolding, which likely reflected a more stressed state for the liver. Additionally, very few genes associated with xenobiotic metabolism were induced when woodrats ingested ecologically relevant plant toxins. Taken together, these

results highlight the important role of temperature on gene expression profiles in the desert woodrat. Cooler temperatures might be a "release state" for the liver, as defined by a greater number of differentially expressed genes from multiple pathways compared to warmer temperatures. These results also help elucidate how ambient temperature alters detoxification and thermoregulatory pathways in mammals. As crucial players in many ecosystems, understanding how herbivorous mammals will respond to temperature and, broadly, climate change, is imperative to creating management plans for their survival.

Introduction

Herbivores confront physiological challenges at every meal; plant material is low in nutrients and high in potentially toxic defense compounds (Freeland and Janzen 1974, Dearing et al. 2005b, Marsh et al. 2006). Although not traditionally included in theories of diet selection, ambient temperature has been demonstrated to have a substantial impact on mammalian herbivores (Dearing et al. 2008, Kurnath and Dearing 2013, Kurnath et al. 2016). Work in pharmacology, agriculture, and more recently ecology has collectively provided support for a phenomenon coined temperature-dependent toxicity (TDT; Keplinger et al. 1959, Spiers et al. 2008, Dearing 2013). Multiple studies have shown that higher temperatures interact with mammalian physiology in a way that results in increased toxicity of foreign compounds (Keplinger et al. 1959, Aldrich et al. 1992, Gordon 1993, Dearing 2013). Behavioral changes have been demonstrated in laboratory rats (Gordon 1993, Gordon et al. 2008), cattle (Aldrich et al. 1992, Spiers et al. 2008), birds (Chatelain et al. 2013), and wild rodents (Dearing et al. 2008, Kurnath and Dearing

2013) in response to TDT. When provided the option, animals challenged by foreign toxins chose to be at cooler ambient temperatures (Gordon et al. 2008) and to ingest nontoxic diets at warmer temperatures (Dearing et al. 2008, Chatelain et al. 2013). Furthermore, increasing temperatures reduced drug clearance times in both laboratory and wild rodents, indicating decreased liver function (Kaplanski and Ben-Zvi 1980, Kurnath and Dearing 2013). Impaired liver function at warmer temperatures could have critical implications for mammalian herbivores that must balance thermoregulation with the detoxification of plant defense compounds in a warming environment.

The specific mechanisms underlying TDT have yet to be elucidated. One possible mode of action resulting in reduced liver function at warmer temperatures could be changes in liver anatomy, such as organ size or vascularization that alters blood flow to this heat-producing organ (Hales et al. 1979, Settivari et al. 2008). Alternatively, enzyme function could alter the efficiency of compound detoxification, whereby there is an overall decrease in enzymatic activity in the liver when animals are at higher ambient temperatures (Kaplanski and Ben-Zvi 1980, Pachecka et al. 1982). Lastly, changes at the molecular level such as differences in gene expression could be driving the observed patterns of increased compound toxicity at warmer ambient temperatures (Settivari et al. 2009). Theses mechanisms are not mutually exclusive and could be acting in concert. For example, blood flow could be diverted away from the liver and towards the periphery at higher temperatures in order to maintain thermal homeostasis. The reduction in blood flow could result in fewer toxins being delivered to the liver and therefore reduced gene expression and enzyme production for detoxification in the liver.

Temperature-mediated shifts in hepatic gene expression were previously documented

by Settivari et al. (2009) in lab rats fed ergot alkaloids, which are fungal toxins known to cause fescue toxicosis in cattle feeding on infected grasses (Aldrich et al. 1992). In this study, genes associated with xenobiotic metabolism, oxidative phosphorylation and antioxidative mechanisms were down-regulated in the liver after a short-term heat-shock treatment, while genes associated with gluconeogenesis and apoptosis were up-regulated (Settivari et al. 2009). This work supports the prediction that gene expression could contribute to TDT; however, temperature treatments in this study differed by 10°C, with the higher temperature being thermally stressful to lab rats (31°C). Heat stress of this magnitude is known to influence hepatic gene expression in the absence of dietary toxins (Feder and Hofmann 1999, Hochachka and Somero 2002, Sonna et al. 2002). The effect of temperature differences outside the bounds of heat stress has received less attention.

Here, we investigated a putative mechanism of TDT by quantifying gene expression changes in the liver of the herbivorous rodent, the desert woodrat (*Neotoma lepida*). Woodrats from the Mojave Desert mainly feed on creosote bush (*Larrea tridentata*, up to 75% of diet) and can ingest doses of toxins in creosote resin that cause kidney cysts and even death in laboratory rodents (Goodman et al. 1970, Karasov 1989). Additionally, gene expression studies previously conducted with *Neotoma* identified candidate hepatic enzymes important for the metabolism of plant toxins, including creosote resin (Dearing et al. 2006, Magnanou et al. 2009, Magnanou et al. 2013), and documented convergent detoxification strategies used by different woodrat species with historical experience with creosote bush (Malenke et al. 2013, Malenke et al. 2014).

In our study, we set out to investigate patterns of hepatic gene expression across temperature and dietary treatments in *N. lepida*. First, we determined the effect of

temperature (22°C versus 27°C) on gene expression while animals ingested a control diet (i.e., without creosote). We predicted overall lower gene expression at warmer temperatures compared to cooler temperatures, based on previous evidence suggesting lower gene expression equated to reduced liver function. Second, we contrasted the effect of diet (control versus creosote) within a temperature treatment (22°C and 27°C). We expected creosote diets to induce detoxification genes, and to a greater extent at 22°C but not at 27°C.

Materials and Methods

Animal Collection and Feeding Trial: Desert woodrats, *Neotoma lepida*, (N=16) were collected from the Mojave Desert near Beaver Dam, Utah (37°06'N, 133°58'W) in May 2012. Sherman live traps were baited with apples, oats, and peanut butter, and cotton batting was provided for nesting material. Creosote leaves were collected from several plants (~10) at the trapping sites for extraction of resin. Plant material was stored on dry ice in the field and at -20°C at the University of Utah (Salt Lake City, UT). Woodrats were transported to an animal facility at the University of Utah, and maintained in shoebox cages (48x27x20 cm) at room temperature (23-25°C) on a 12L:12D cycle. Woodrats were provided rabbit chow pellets (Teklad 2031 formula, Harlan Laboratories, Madison WI) and water *ad libitum* for at least three weeks before experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC #12-12010). Woodrats were divided evenly into four groups, balanced for sex, and exposed to four different treatments of temperature and diet (explained below).

Two temperature treatments were used in the current study: one within the thermal neutral zone of these animals (27°C), hereafter "thermoneutral", while the other was below the thermal neutral zone (22°C), hereafter "cool". Similar temperatures have been used previously to investigate temperature-dependent responses in *Neotoma* species (McLister et al. 2004, Dearing et al. 2008, Kurnath and Dearing 2013, Kurnath et al. 2016). Moreover, these temperatures reflected physiologically and ecologically relevant temperatures for *N. lepida* from the Mojave Desert (Kurnath and Dearing 2013). Each temperature treatment was maintained in separate rooms with wall thermostats and space heaters (DeLonghi, Upper Saddle River NJ). Room temperatures were measured every 10 minutes with HOBO data loggers (UA-001-08 pendant, Onset, Bourne MA) prior to and during experiments. Animals were acclimated to either 27°C or 22°C for at least 14 days, similar to previous studies (McLister et al. 2004, Dearing et al. 2008, Kurnath et al. 2016).

Animals were presented two diet treatments at each temperature treatment, either a powdered rabbit chow (Harlan Laboratories) without creosote resin (i.e., control treatment) or powdered rabbit chow amended with creosote bush resin (i.e., creosote treatment). Animals in the control treatments received powdered rabbit chow with 0% resin for all eight days of the trial. Resin was extracted and diets were prepared as in Magnanou et al. (2009). Diets were prepared before the feeding trial and stored at -20°C. Animals were fed a gradually increasing amount of resin over an eight-day period to allow induction of biotransformation enzymes (Klaassen 2001). Animals in the creosote treatments received powdered rabbit chow with 0% resin for four days, an intermediate dose of resin for two days, followed by 4% resin for the cool-temperature treatment

(22°C) or 7% resin at the thermoneutral temperature treatment (27°C) for the final two days. Different resin concentration of the diets were given to control for different daily food intakes of animals at the two temperatures (Kurnath et al. 2016), in order to ensure all animals ingested on average 0.3 g of creosote resin per day. Creosote resin concentrations were chosen based on previous work (Kurnath et al. 2016); however, in this study the animals did not differ significantly in food intake and we were unable to generate similar resin doses across temperature treatments (Table 3.1). During the feeding trials, woodrats had *ad libitum* access to water and food, which were replenished daily. Food intake, creosote resin intake, and body mass were measured daily. Mean body mass and food intake per gram body mass from the last two days of the trial were compared across all four treatment groups using two-way ANVOAs with post hoc Tukey HSD tests, with diet and temperature as independent variables. Mean resin intake per gram body mass from the last two days of the trial was compared across temperature groups using a one-way ANOVA.

At the end of the feeding trial, animals were dispatched using CO_2 asphyxiation. Liver weight was measured and compared across groups with two-way ANOVAs and post hoc Tukey HSD tests, with diet and temperature as independent variables. Liver tissue was preserved in RNAlater (Sigma) overnight at 4°C. Excess RNAlater was poured off and the tissue was archived at -80°C. RNA was extracted (RNAqueous, Ambion) from the samples and sent to the University of Utah Microarray Core Facility for the microarray analysis.

Microarray Analysis: Liver samples were analyzed using a custom microarray built from a liver transcriptome (Roche 454 platform) of the desert woodrat (Malenke et al.

2013). Details on the design of the custom microarray (eArray, Agilent) and processing methods can be found in (Malenke et al. 2013, Malenke et al. 2014). Each RNA sample was hybridized to a separate array, resulting in biological duplicates for each temperature and diet combination. All raw microarray data were deposited in Gene Expression Omnibus (NCBI, series record GSE80595) in accordance with minimum information about microarray experiment compliance (MIAME).

Microarray features were extracted using AgilentFilter (BioInformatic Shared Resource, Huntsman Cancer Institute and University of Utah) as previously described (Malenke et al. 2013, Malenke et al. 2014). Briefly, all control spots, non-uniform spots and population outlier spots were removed from the data set and intensity values were $\log_2$-transformed. Intensity data from duplicate probes were combined into unique probe groups. Spot intensity data from different probes with the same annotation information were maintained separately because it is possible that the original assembled contigs, from which the probes were designed, were from different genetic loci despite the shared match to a rodent gene annotation. The performance of the custom *Neotoma* expression array was previously validated (Malenke et al. 2013).

Comparisons of Gene Expression: Spot intensity data from the *Neotoma* microarray were batch uploaded to GeneSifter 3.7 (Geospiza, PerkinElmer). Prior to comparing expression profiles, the consistency of transcriptome response was evaluated by comparing overall gene expression profiles across individuals. After normalizing intensity data, individuals were clustered by gene expression using all woodrat-derived probes (N=6,286). Clustering parameters were distance:correlation, linkage:average, row centered:by genes.

Multiple pairwise comparisons were generated in GeneSifter to compare gene expression. In all comparisons, the quality requirement was set to 1 and all individuals from both treatments were required to pass. T-tests were performed (alpha=0.05) and the resulting gene/probe lists were ranked by fold change ($\geq$2). This method is less conservative than applying a false discovery rate correction (Benjamini and Hochberg 1995), yet fold change ranking is more consistent (Guo et al. 2006).

A total of four pair-wise comparisons were conducted in GeneSifter (Figure 3.1). To investigate the effect of temperature, animals at 27°C were compared to animals at 22°C while ingesting the nontoxic, control diets. We ran two comparisons to investigate the effect of diet: one comparison with animals at 22°C and one comparison with animals at 27°C. Within a temperature treatment, animals feeding on the creosote diet were compared to animals fed the control diet. Lastly, animals at 27°C were compared to animals at 22°C while ingesting diets with creosote resin. The lists of significantly different genes were ordered by fold change amount and direction. Transcripts were described as up-regulated at either the thermoneutral (27°C) or cool (22°C) temperature. In addition, transcripts that were up-regulated on the creosote diet compared to the control diet were considered genes that were induced by creosote resin.

Treatments were compared by examining the number of annotated, up-regulated transcripts as well as the KEGG pathways and Gene Ontology (GO) terms for biological processes represented by those transcripts. Z-scores were used to determine significant representation in these functional assays. KEGG pathways or GO terms with z-scores >2 were considered to be significantly overrepresented in the results; that is, the genes in that ontology were more likely to be up-regulated than expected by chance, given the number

features on the array within that particular GO term. The number of transcripts, fold change values and annotation of the hybridized probe were reported for each comparison. We also identified and categorized transcripts based on potential function from GO terms and KEGG pathways.

Results

Feeding Trial: Diet significantly affected total food intake; however there was no effect of temperature, nor an interaction effect (Table 3.1). Woodrats fed control diets always ate more food than woodrats fed creosote diets, regardless of temperature. Animals at 27°C ingested ~11% less food on both diets compared to woodrats at 22°C, but this difference was not significant (Table 3.1). There was no effect of temperature or diet on starting or ending body mass (Table 3.1), and all woodrats maintained body mass throughout the eight-day feeding trial (paired t-test, $p=0.8$). Similarly, there was no effect of temperature or diet on liver mass of woodrats (Table 3.1), even though average liver size at 22°C was 14% larger in animals ingesting the creosote diet compared to animals ingesting the control diet. Animals at 22°C appeared to have larger liver masses relative to body mass, but a Tukey's post hoc test did not reveal significant differences across the four treatment groups (Table 3.1).

Microarray Quality Control: All of the 16 arrays passed at least six of Agilent's nine quality metrics. Three samples passed 7 metrics, nine samples passed 8 metrics, and three samples passed all 9 quality metrics. All samples were kept in the analysis. On average, less than 0.05% of the features across all arrays were flagged as nonuniform. The clustergram grouping individual woodrats by overall expression profile (based on all

6,286 probes) grouped woodrats from the "cool, creosote" treatment in one monophyletic clade (Figure 3.2). The remaining individuals did not group by treatment but seemed to group by temperature. Woodrats at 27°C generally grouped together regardless of dietary treatment.

General Findings: A total of 107 unique transcripts were differentially expressed across all four pair-wise comparisons conducted in the present study. Of these results, only 10 transcripts appeared in more than one comparison (Figure 3.3). Two transcripts were associated with detoxification (p450 oxidoreductase and glutathione S-transferase mu 2) and two transcripts were associated with hepatocellular carcinomas (annexin A3 and histidine triad nucleotide binding protein 2). Annexin A3 displayed the greatest fold change (4.5, induced on creosote diet compared to control diet at 22°C) out of these 10 transcripts. Other transcripts included Alkbh2, a primary housekeeping DNA demethylase responsible for repairing DNA; F11 coagulation factor XI, in which a deficiency will lead to bleeding disorders; two transcripts from the 134b family; and Cyp26a1, from the family of cytochrome p450s that regulates cellular levels of retinoic acid, which is a metabolite of vitamin A.

Transcripts Influenced by Temperature: Temperature influenced the number of differentially expressed transcripts in woodrats ingesting a control diet (Figure 3.1, comparison A). On the control diet, 15 transcripts were significantly up-regulated at 22°C (i.e., down-regulated at 27°C), while only 8 transcripts were significantly up-regulated at 27°C (Table 3.2). In addition, more transcripts associated with detoxification genes were up-regulated at 22°C compared to 27°C (Table 3.2). A transcript variant of Tsukushi displayed the highest fold change at 22°C (Tsku, 5.43), and a member of the

tumor necrosis factor receptor superfamily had the highest fold change at 27°C (Tnfsrf18, 2.89; Table 3.3).

Genes up-regulated at 22°C seem to have different functions compared to genes up-regulated at 27°C. Genes involved with repair and regrowth were up-regulated at 22°C, including DNA repair (Alkbh2), steroid biosynthesis (Hint2), metabolism (Baat, Pmm1, Phyh), and organ regeneration (Eif4a1). Oxidative-reduction reactions (Phyh, Alkbh2), cell adhesion (Itga), and signaling (Tsku, Itgae) were molecular processes also notably up-regulated at 22°C. Conversely, at least two genes significantly up-regulated at 27°C were involved with cell death and degradation: Tnfsrf10b (apoptosis) and Snx16 (targeting for lysosome). Other potential functions of genes up-regulated at 27°C include binding (Scgbc1), ER and extracellular region (Scgbc1, Lmf2), transferase activity (Ugt2b34) and signal transduction (Tnfrsf18, Tnfrsf10b).

More KEGG pathways were overrepresented in animals eating a control diet at 27°C compared to 22°C (14 versus 8 pathways; Table 3.4). Regardless of temperature, 16 out of 22 KEGG pathways listed were associated with metabolism. Most pathways at 22°C were associated with bile acid coenzyme A (Baat), while at 27°C most pathways were associated with UDP glucuronosyltransferase (Ugt2b34), a detoxification enzyme. No GO terms contained gene lists with >10 transcripts and were not reported.

Temperature also appears to greatly impact differential expression in the liver when transcript numbers were compared between two other pair-wise comparisons (Figure 3.1, comparison B). In two comparisons testing the effect of diet, one at 22°C and one at 27°C, more transcripts were differentially expressed at 22°C compared to 27°C (49 versus 10, Tables 3.5 and 3.6). Additionally, more KEGG pathways were overrepresented in the

22°C comparison versus the 27°C comparison (Tables 3.7 and 3.8).

Transcripts Induced and Repressed by Creosote: Two within-temperature treatment comparisons were conducted to investigate the impact of diet on gene expression in the liver (Figure 3.1, comparison B). There was one transcript that appeared in both comparisons and was induced by creosote in both instances, Cyp26a1.

At 22°C, 49 transcripts were differentially expressed, with 29 genes induced by creosote and 20 genes repressed by creosote (Table 3.5). Very few genes at 22°C were associated with detoxification: only one induced gene (Gstm1, 2.04) and two repressed genes (Por, 2.01 and Fmo3, 2.08; Table 3. 9) that also had low fold change values. The transcript with the highest fold change value was annexin A3 (4.5; Table 3.9), which was induced on creosote and involved with phagocytosis.

A greater number of GO terms induced by creosote resin were overrepresented at 22°C compared to GO terms repressed by creosote, by a difference of three-fold (35 versts 13; Table 3.10). Overrepresented KEGG pathways showed a similar pattern, by a difference of six-fold (26 induced pathways versus 4 repressed pathways, Table 3.7). In addition, the listed GO term and KEGG pathways were associated with xenobiotic metabolism and detoxification. More than half of the KEGG pathways induced by creosote resin were categorized as human disease related (15 pathways), which encompassed 12 unique transcripts.

Transcripts listed as differentially expressed by diet at 22°C were not specific to biotransformation but were associated with numerous, general processes within the cell. For instance, transcripts involved with cell adhesion (Itgb7, Cd9), cytoskeleton organization (Tmsb4x), and cell migration (Anxa3) were all induced by creosote. Only

one transcript pertaining to xenobiotic metabolism (Gstm2) was induced by creosote. In addition, transcription-related processes (Srebf1, Hmgb2l1, Fli1, Sik1), protein phosphorylation (Il1b, Lck, Aif1), and the regulation of various enzymatic activity (RT1-Da, Pla2g7, Pcolce, Serpinb6a) were significantly induced by creosote resin at 22°C. Lastly, creosote resin also induced two immune response transcripts (Procr, Ly86) and one associated with apoptosis (Bcl2a1d). Transcripts related to pain sensory, response to stress, transcription, and DNA repair were also repressed by creosote at 22°C (Fam134b, Alkbh2, Trim24). There were many processes with only one associated transcript that were repressed by creosote, and these included the innate immune response, lipid transport, blood coagulation, histone deacetylation, ubiquitin-dependent process, Ras protein signaling, and kidney development. Taken together, genes influenced by dietary creosote at 22°C were not associated with detoxification but many other, diverse processes.

Substantially fewer transcripts were induced and repressed by dietary creosote at 27°C compared to 22°C (Tables 3.6 and 3.11). Only five transcripts were induced and five different transcripts were repressed at 27°C for *N. lepida*. None of the transcripts were involved with xenobiotic metabolism. The highest fold change value belonged to D-site of albumin promoter (Dbp, 5.38; Table 3.11), which was repressed on creosote and plays an important role in cell survival, proliferation and differentiation. There were no GO terms that contained gene lists of >10 transcripts in this pairwise comparison. In addition, significantly fewer KEGG pathways were overrepresented at 27°C compared to 22°C (12 versus 30 total KEGG pathways; Tables 3.7 and 3.8). Most KEGG pathways induced by creosote were associated with carbohydrate metabolism. Lastly, a majority of

the transcripts and KEGG pathways listed in this comparison were associated with gluconeogenesis, glycolysis and glucose/glycogen pathways. For instance, creosote significantly induced Hint2 and G6pc but also repressed Fbp2 and Gys2 at 27°C.

Transcripts Affected by Temperature and Creosote: Similar to other comparisons, more differentially expressed transcripts were found to be up-regulated at 22°C compared to 27°C in woodrats presented diets amended with creosote resin (Figure 3.1, comparison C; Table 3.12). In addition, this comparison resulted in the highest number of transcripts associated with detoxification were differentially expressed (7 transcripts at 27°C and 13 transcripts at 22°C; Table 3.13). There were also more GO terms and KEGG pathways significantly overrepresented at 22°C compared to at 27°C (Tables 3.14 and 3.15). However, these results were confounded by the fact that woodrats ingested unequal amounts of creosote resin while housed at 22°C and 27°C (Table 3.1); therefore, observed differences could be due to temperature treatments, dietary creosote, or an interaction between the two factors.

Discussion

While there has been mounting evidence for temperature-dependent toxicity (TDT) in model and nonmodel systems, the underlying mechanism of how toxicity increases at warmer ambient temperatures has yet to be elucidated. In the present study, we investigated the effect of temperature and dietary toxins on patterns of relative gene expression in the liver of desert woodrats, *Neotoma lepida*. We found that cooler ambient temperatures (22°C) resulted in a greater number of differentially expressed genes compared to a slightly warmer, thermoneutral temperature (27°C). However,

dietary toxins did not induce a substantial number of genes associated with xenobiotic metabolism, regardless of temperature. Surprisingly, more detoxification-specific genes appeared in comparisons testing the influence of temperature than in comparisons testing the effect of diet. Taken together, our results suggest that small changes in ambient temperature can cause limited differential gene expression in the livers of mammalian herbivores. It should be noted that our analysis focused on changes in relative gene expression, and it is likely that absolute or constitutive differences were not revealed. It is impossible to identify genes expressed at similar levels across treatments when analyzing differential gene expression.

Firstly, it is important to recognize the relatedness of individual woodrats across treatment groups in the trial. The group ingesting creosote resin at 22°C was the treatment group most similar to itself and also the most different from all other individuals in experiment (Figure 3.2). The clustering of these individuals suggests that their differential gene expression responses are very similar. We theorize more below about gene profiles could define this treatment group. Additionally, individuals from both thermoneutral treatment groups (i.e., control and creosote diets) tended to cluster together, suggesting that these groups behave in a similar manner. Further support for this conclusion can be found in the short list of differentially expressed genes between the control and creosote treatment groups at 27°C (Table 3.6) compared to the genes listed at 22°C (Table 3.5). Fewer differentially expressed genes suggest there are fewer changes between these two treatment groups. The clustergram provided a unique perspective on the influence of temperature on gene expression in the liver of woodrats.

A 5°C change in ambient temperature resulted in both expected and unexpected

changes in differential gene expression for animals ingesting a nontoxic diet (Figure 3.1, comparison A). More transcripts were differentially expressed at 22°C than 27°C, supporting our prediction that lower gene expression at warmer temperatures could explain reduced liver function. However, an unexpected result was the overrepresentation of KEGG pathways related to detoxification at 27°C (Table 3.8). Many KEGG pathways up-regulated at 27°C were associated with a signal gene, UDP glucuronosyltransferase (Ugt2b34). This family of conjugating enzymes is important for metabolizing creosote resin in *Neotoma* species (Mangione et al. 2000, Lamb et al. 2001, Mangione et al. 2001, Haley et al. 2008, Malenke et al. 2014). UGTs are also very versatile and can conjugate endogenous substrates such as steroids and bilirubin (Klaassen 2001). The up-regulation of Ugt2b34 at 27°C compared to 22°C could be indicative of an increased need for endogenous glucuronidation. If such is the case, then these animals utilized UGTs while ingesting a control diet, and this use could potentially limit their ability to employ UGTs when challenged by creosote resin.

Our results suggest that molecular-level changes can occur in the liver after a small increase in temperature, with potentially more growth at 22°C and more stress at 27°C. For instance, genes associated with steroid biosynthesis (histidine triad nucleotide binding protein 2), DNA repair (alkylation repair homolog 2), and metabolism (bile acid coenzyme A: amino acid N-acyltransferase) were up-regulated at 22°C compared to 27°C. These genes could be indicative of biosynthesis and may represent a building state for the liver at 22°C. In contrast, genes associated with apoptosis (tumor necrosis factor receptor superfamily 18 and 10b) were significantly up-regulated at 27°C compared to 22°C. Increased regulation of apoptosis processes was previously reported in a study

testing the effects of short-term heat shock in mice challenged with toxins (Settivari et al. 2009). Tumor necrosis factors have also been described as heat-shock-related genes and were up-regulated in a study investigating chronic heat exposure in chickens (Li et al. 2011). Additionally, protein misfolding genes were up-regulated at 27°C compared to 22°C (Serine peptidase inhibitor A3C), which was a pattern previously documented in lab rats exposed to short-term heat stress (Stallings et al. 2014). Overall, these gene descriptions, combined with the overrepresented KEGG pathways involving metabolism and immune system responses, could imply that 27°C is a relatively more stressful state than 22°C for woodrats.

The effect of dietary creosote on differential gene expression in the liver of woodrats housed either at 22°C or 27°C did not support our predictions (Figure 3.1, comparison B). Very few detoxification-specific transcripts were differentially expressed at 22°C, no detoxification transcripts were differentially expressed at 27°C, and there were no xenobiotic-specific KEGG pathways listed as overrepresented at either 22°C or 27°C. The lack of detoxification was surprising since plant secondary compounds such as alkaloids and terpenes induce a drug metabolism response in both laboratory and wild rodents (Settivari et al. 2006, Skopec et al. 2007, Magnanou et al. 2009). Yet our results were in accordance with a more recent *Neotoma* study, which found that dietary toxins did not significantly affect xenobiotic metabolism (Malenke et al. 2014). A similar trend was documented in a study investigating gene expression in insect herbivores, in that glucosinolates did not induce phase I or phase II detoxification genes as predicted but instead induced stress-related genes and pathways (Whiteman et al. 2012). Additionally, there was no overlap in genes induced by creosote between 22°C and 27°C (Tables 3.9

and 3.11). The lack of similarity across induced genes suggested that woodrats may be mounting diverse responses to dietary creosote at different ambient temperatures.

Variation in differential gene expression due to creosote at 22°C compared to 27°C may be an example of temperature constraints that could challenge herbivorous mammals. Animals could be energy limited or in an overall stressful state at warmer temperatures, since genes and KEGG pathways associated with gluconeogenesis were significantly influenced by creosote at 27°C. Differing parts of the gluconeogenesis pathway were both induced and repressed by creosote (Table 3.8), likely due to changes in the complicated, multistep formation of glucose. Other work has documented gluconeogenesis as a significantly altered pathway in lab rats affected by diet and temperature (Settivari et al. 2006, Settivari et al. 2009). Likewise, the expression of mitochondrial energy synthesis genes were significantly influenced by ambient temperature in Japanese quail (Voltolini et al. 2014). Conversely, cooler temperatures could represent a "release state" in the liver. More genes were differentially expressed due to dietary creosote at 22°C compared to 27°C by almost five-fold (Tables 3.5 and 3.6). In addition, half of the overrepresented KEGG pathways induced by creosote at 22°C were linked to lysosome and intraorganelle membrane function (RT1-Da), which could be a sign of high cellular turnover. Lastly, 15 out of 26 overrepresented KEGG pathways were associated with human disease (Table 3.7), which could indicate the liver has the flexibility to turn on numerous genes and pathways at 22°C.

Patterns in differential gene expression were apparent in woodrats consuming creosote resin, although animals were not ingesting the same total amount of creosote (Figure 3.1, comparison C). Animals at 27°C consumed 57% more creosote than animals

at 22°C (Table 3.1). Regardless, more transcripts were differentially expressed at 22°C compared to 27°C, including a high number of detoxification-specific transcripts (Table 3.13), which is consistent with our other comparisons of treatment groups. In addition, the transcript profile of differentially expressed genes at 22°C was very similar to genes previously identified by Malenke (2014). Moreover, fold changes for transcripts at 22°C were the highest values recorded in our analysis, with a glutathione S-transferase mu 7 displaying a 6.15 fold change (Table 3.13). Higher fold changes could be indicative of substantial modifications in the liver for animals at 22°C while ingesting creosote resin, which further supports the idea that cooler temperatures do not restrict gene expression in the liver. Lastly, despite animals at 27°C facing a bigger toxic challenge, woodrats at the thermoneutral temperature were not able to mount a response to dietary toxins like animals at 22°C. This pattern in differential gene expression provides additional evidence that fewer genes can be turned on at warmer temperatures compared to cooler temperatures.

Even though detoxification genes were differentially expressed at both 22°C and 27°C while woodrats ingested creosote, transcripts were from different enzyme families. For instance, glutathione S-transferases, aldehyde dehydrogenases, and carboxylesterases were up-regulated at 22°C, while various transferases were up-regulated at 27°C, including sulfotransferases; however, UGTs were notably absent (Table 3.13). While purely speculative, it is possible that sulfotransferases were up-regulated at 27°C because UGTs were preferentially utilized at warmer temperatures while woodrats were feeding on control diets. Regardless of the caveats associated with this comparison, our results shed light on the effects that temperature and diet can have on hepatic gene expression.

The temperature-mediated changes in differential gene expression we found were similar to those previously documented in fish studies, even though these animals differ in thermal physiology from endothermic mammals. The current literature mainly encompasses temperature effects in ectotherms due to their more-or-less direct behavioral and physiological response to heat (i.e., Bale et al. 2002). Additionally, a pivotal paper by Podrabsky and Somero (2004) stated that constant versus fluctuating temperatures altered gene expression in fish. They found that the control of cell growth and proliferation were constant and central responses to temperature, but other pathways became important based on controlled or fluctuating temperatures, such as carbohydrate metabolism and membrane structure maintenance (Podrabsky and Somero 2004). Furthermore, a recent chemical toxicology study in fish found that cadmium toxicity increased at higher temperatures (Vergauwen et al. 2013). The underlying mechanism was likely due to a change in toxin sensitivity via accumulation of cadmium in the tissue rather than direct liver oxidative damage (Vergauwen et al. 2013). However, the group also found that gluconeogenesis genes were good markers of cold-induced oxidative stress (Vergauwen et al. 2013). These studies highlight the potential that temperature and toxins could be problematic for numerous animal species, regardless of their thermoregulatory strategy.

Our results did support the prediction that fewer genes would be expressed at higher ambient temperatures, but also lend to new speculation about the mechanism of TDT. Changes in blood flow could be partially responsible, because blood flow is known to be diverted to the periphery and away from the viscera as a thermoregulatory response in mammals at warmer temperatures (Grayson et al. 1966, Dearing 2013). However, blood-

shunting is an immediate, physiological response to thermal stress that is often short-term and potentially energetically expensive (Hales et al. 1979, Reichard et al. 2010). It is probable that multiple pathways pertaining to thermoregulation and detoxification are acting in concert, ultimately resulting in TDT. Future avenues of research could include measuring liver damage with conventional assays, such as serum levels of alanine transaminase (Kim et al. 2008, Ozer et al. 2008), to better define hepatic stress. In addition, monitoring core body temperature across dietary and temperature treatments could provide helpful information regarding the environment in which the liver is functioning (Rothwell and Stock 1980, Settivari et al. 2006).

Lastly, our results should be considered within the context of global climate change due to the implications of TDT and potential effects on ecologically and evolutionarily relevant systems. While animals from diverse environments have different ranges of thermal tolerance and thus varying abilities to adapt to climate change (Tomanek 2008), mammalian herbivores of all shapes and sizes will likely face the combined challenge of dietary toxins and rising ambient temperature. In addition, the historically undocumented changes in climate will undoubtedly play an important role in how mammalian herbivores will respond to TDT (Field et al. 2014), which in turn could affect their foraging behavior and ability to survive in a warming climate. Thus, understanding the mechanistic nature of TDT will advance the field of herbivore ecology and also inform predictions about mammalian species' responses to changes in their natural environment.

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References

Aldrich, C. G., J. A. Paterson, J. L. Tate, and M. S. Kerley. 1992. The effects of endophyte-infected tall fescue consumption on diet utilization and thermal regulation in cattle. Journal of Animal Science 71:164-170.

Bale, J. S., G. J. Masters, I. D. Hodkinson, C. Awmack, T. M. Bezemer, V. K. Brown, J. Butterfield, A. Buse, J. C. Coulson, J. Farrar, J. E. G. Good, R. Harrington, S. Hartley, T. H. Jones, R. L. Lindroth, M. C. Press, I. Symrnioudis, A. D. Watt, and J. B. Whittaker. 2002. Herbivory in global climate change research: direct effects of rising temperature on insect herbivores. Global Change Biology 8:1-16.

Benjamini, Y. and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological) 57:289-300.

Chatelain, M., C. G. Halpin, and C. Rowe. 2013. Ambient temperature influences birds' decisions to eat toxic prey. Animal Behaviour 86:733-740.

Dearing, M. D. 2013. Temperature-dependent toxicity in mammals with implications for herbivores: a review. Journal of Comparative Physiology B 183:43-50.

Dearing, M. D., W. J. Foley, and S. McLean. 2005. The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. Annual Review of Ecology, Evolution, and Systematics 36:169-189.

Dearing, M. D., J. S. Forbey, J. D. McLister, and L. Santos. 2008. Ambient temperature influences diet selection and physiology of an herbivorous mammal, *Neotoma albigula*. Physiological and Biochemical Zoology 81:891-897.

Dearing, M. D., M. M. Skopec, and M. J. Bastiani. 2006. Detoxification rates of wild herbivorous woodrats (*Neotoma*). Comparative Biochemistry and Physiology A Mol Integr Physiol 145:419-422.

Feder, M. E. and G. E. Hofmann. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annual Review of Physiology 61:243-282.

Field, C. B., V.R. Barros, D.J. Dokken, K.J. Mach, M.D. Mastrandrea,, M. C. T.E. Bilir, K.L. Ebi, Y.O. Estrada, R.C. Genova, B. Girma, E.S. Kissel, A.N. Levy, S. MacCracken,, and a. L. L. W. e. P.R. Mastrandrea, editors. 2014. Climate Change 2014: Impacts, Adapation, and Vulnerability. Cambridge University Press, New York, NY.

Freeland, W. J. and D. H. Janzen. 1974. Strategies in Herbivory by Mammals: The Role of Plant Secondary Compounds. American Naturalist 108:269-289.

Goodman, T., H. Grice, G. Becking, and F. Salem. 1970. A cystic nephropathy induced by nordihydroguaiaretic acid in the rat. Light and electron microscopic investigations. Laboratory Investigation; a Journal of Technical Methods and Pathology 23:93-107.

Gordon, C. 1993. Temperature regulation in laboratory rodents. Cambridge University Press, New York, NY.

Gordon, C. J., P. J. Spencer, J. Hotchkiss, D. B. Miller, P. M. Hinderliter, and J. Pauluhn. 2008. Thermoregulation and its influence on toxicity assessment. Toxicology 244:87-97.

Grayson, J., M. Irvine, and T. Kinnear. 1966. Observations on temperature distribution in the cardiovascular system, thorax and abdomen of monkeys in relation to environment. The Journal of Physiology 184:581-593.

Guo, L., E. K. Lobenhofer, C. Wang, R. Shippy, S. C. Harris, L. Zhang, N. Mei, T. Chen, D. Herman, and F. M. Goodsaid. 2006. Rat toxicogenomic study reveals analytical consistency across microarray platforms. Nature Biotechnology 24:1162-1169.

Hales, J., L. Rowell, and R. King. 1979. Regional distribution of blood flow in awake heat-stressed baboons. American Journal of Physiology-Heart and Circulatory Physiology 237:H705-H712.

Haley, S. L., J. G. Lamb, M. R. Franklin, J. E. Constance, and M. D. Dearing. 2008. "Pharm-ecology" of diet shifting: biotransformation of plant secondary compounds in creosote (*Larrea tridentata*) by a woodrat herbivore, *Neotoma*

lepida. Physiological and Biochemical Zoology 81:584-593.

Hochachka, P. and G. Somero. 2002. Biochemical Adaptation, Mechanism and Process in Physiological Evolution. Oxford University Press, New York.

Kaplanski, J. and Z. Ben-Zvi. 1980. Effect of chronic heat exposure on in-vitro drug metabolism in the rat. Life Sciences 26:639-642.

Karasov, W. H. 1989. Nutritional bottleneck in a herbivore, the desert wood rat (Neotoma lepida). Physiological Zoology 62:1351-1382.

Keplinger, M. L., G. E. Lanier, and W. B. Deichmann. 1959. Effects of environmental temperature on the acute toxicity of a number of compounds in rats. Toxicology 1:156-161.

Kim, W. R., S. L. Flamm, A. M. Di Bisceglie, and H. C. Bodenheimer. 2008. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. Hepatology 47:1363-1370.

Klaassen, C. 2001. Casarett and Doull's Toxicology: The Basic Science of Poisons. McGraw Hill, New York.

Kurnath, P. and M. D. Dearing. 2013. Warmer ambient temperatures depress liver function in a mammalian herbivore. Biology Letters 9.

Kurnath, P., N. D. Merz, and M. D. Dearing. 2016. Ambient temperatures affect tolerance to plant secondary compounds in a mammalian herbivore. Proceedings of the Royal Society B 283.

Lamb, J. G., J. S. Sorensen, and M. D. Dearing. 2001. Comparison of detoxification enzyme mRNAs in woodrats (*Neotoma lepida*) and laboratory rats. Journal of Chemical Ecology 27:845-857.

Li, C., X. Wang, G. Wang, N. Li, and C. Wu. 2011. Expression analysis of global gene response to chronic heat exposure in broiler chickens (*Gallus gallus*) reveals new reactive genes. Poultry Science 90:1028-1036.

Magnanou, E., J. R. Malenke, and M. D. Dearing. 2009. Expression of biotransformation genes in woodrat (*Neotoma*) herbivores on novel and ancestral diets: identification of candidate genes responsible for dietary shifts. Molecular Ecology 18:2401-2414.

Magnanou, E., J. R. Malenke, and M. D. Dearing. 2013. Hepatic gene expression in herbivores on diets with natural and novel plant secondary compounds. Physiological Genomics 45:774-785.

Malenke, J. R., B. Milash, A. W. Miller, and M. D. Dearing. 2013. Transcriptome sequencing and microarray development for the woodrat (*Neotoma* spp.): custom genetic tools for exploring herbivore ecology. Molecular Ecology Resources 13:674-687.

Malenke, J. R., M. M. Skopec, and M. D. Dearing. 2014. Evidence for functional convergence in genes upregulated by herbivores ingesting plant secondary compounds. BMC Ecology 14:23.

Mangione, A. M., D. Dearing, and W. Karasov. 2001. Detoxification in relation to toxin tolerance in desert woodrats eating creosote bush. Journal of Chemical Ecology 27:2559-2578.

Mangione, A. M., M. D. Dearing, and W. H. Karasov. 2000. Interpopulation differences in tolerance to creosote bush resin in desert woodrats (*Neotoma lepida*). Ecology 81:2067-2076.

Marsh, K. J., I. R. Wallis, R. L. Andrew, and W. J. Foley. 2006. The Detoxification Limitation Hypothesis: Where Did it Come From and Where is it Going? J Chem Ecol 32:1247-1266.

McLister, J., J. Sorensen, and M. Dearing. 2004. Effects of consumption of juniper (Juniperus monosperma) on cost of thermoregulation in the woodrats Neotoma albigula and Neotoma stephensi at different acclimation temperatures. Physiol Biochem Zool 77:305-312.

Ozer, J., M. Ratner, M. Shaw, W. Bailey, and S. Schomaker. 2008. The current state of serum biomarkers of hepatotoxicity. Toxicology 245:194-205.

Pachecka, J., K. Kobylińska, H. Miaskiewicz, and W. Bicz. 1982. Hepatic microsomal mixed-function oxidases in rats exposed to high ambient temperature. Acta Physiologica Polonica 34:563-568.

Podrabsky, J. E. and G. N. Somero. 2004. Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish Austrofundulus limnaeus. Journal of Experimental Biology 207:2237-2254.

Reichard, J. D., S. I. Prajapati, S. N. Austad, C. Keller, and T. H. Kunz. 2010. Thermal Windows on Brazilian Free-tailed Bats Facilitate Thermoregulation during Prolonged Flight. Integrative Comparative Biology 50:358-370.

Rothwell, N. J. and M. J. Stock. 1980. Similarities between cold- and diet-induced thermogenesis in the rat. Canadian Journal of Physiology and Pharmacology 58:842-848.

Settivari, R., T. Evans, P. Eichen, G. Rottinghaus, and D. Spiers. 2008. Short-and long-term responses to fescue toxicosis at different ambient temperatures. Journal of Thermal Biology 33:213-222.

Settivari, R., T. Evans, L. Yarru, P. Eichen, P. Sutovsky, G. Rottinghaus, E. Antoniou, and D. Spiers. 2009. Effects of short-term heat stress on endophytic ergot alkaloid-induced alterations in rat hepatic gene expression. Journal of Animal Science 87:3142-3155.

Settivari, R. S., S. Bhusari, T. Evans, P. A. Eichen, L. B. Hearne, E. Antoniou, and D. E. Spiers. 2006. Genomic analysis of the impact of fescue toxicosis on hepatic function. Journal of Animal Science 84:1279-1294.

Skopec, M. M., S. Haley, and M. D. Dearing. 2007. Differential hepatic gene expression of a dietary specialist (*Neotoma stephensi*) and generalist (*Neotoma albigula*) in response to juniper (*Juniperus monosperma*) ingestion. Comparative and Biochemical Physiology Part D Genomics Proteomics 2:34-43.

Sonna, L. A., J. Fujita, S. L. Gaffin, and C. M. Lilly. 2002. Invited review: effects of heat and cold stress on mammalian gene expression. Journal of Applied Physiology 92:1725-1742.

Spiers, D. E., T. J. Evans, and G. E. Rottinghaus. 2008. Interaction between thermal stress and fescue toxicosis: Animal models and new perspectives.*in* C. P. W. C.A. Roberts, D.E. Spiers, editor. Neotyphodium in Cool-Season Grasses. Blackwell Publishing Ltd, Oxford, UK.

Stallings, J. D., D. L. Ippolito, V. Rakesh, C. E. Baer, W. E. Dennis, B. G. Helwig, D. A. Jackson, L. R. Leon, J. A. Lewis, and J. Reifman. 2014. Patterns of gene expression associated with recovery and injury in heat-stressed rats. BMC Genomics 15:1058.

Tomanek, L. 2008. The importance of physiological limits in determining biogeographical range shifts due to global climate change: the heat-shock response. Physiological and Biochemical Zoology 81:709-717.

Vergauwen, L., A. Hagenaars, R. Blust, and D. Knapen. 2013. Temperature dependence of long-term cadmium toxicity in the zebrafish is not explained by liver oxidative stress: evidence from transcript expression to physiology. Aquatic Toxicology 126:52-62.

Voltolini, D., A. Del Vesco, E. Gasparino, S. Guimarães, A. O. Neto, E. Batista, and A. Ton. 2014. Mitochondrial gene expression in the liver and muscle of high and low feed efficiency Japanese quail layers subjected to different environmental temperatures. Genetics and Molecular Research: GMR 13:4940-4948.

Whiteman, N. K., A. D. Gloss, T. B. Sackton, S. C. Groen, P. T. Humphrey, R. T. Lapoint, I. E. Sønderby, B. A. Halkier, C. Kocks, F. M. Ausubel, and N. E. Pierce. 2012. Genes Involved in the Evolution of Herbivory by a Leaf-Mining, Drosophilid Fly. Genome Biology and Evolution 4:900-916.

Table 3.1: Comparisons (one- or two-way ANOVAs) of data collected during 8-day feeding trial across four experimental treatments of woodrats (N=16 total). Results recorded as means ± S.E. values. P values of ≤0.05 are **bolded**.

	Diet Treatment		**Temperature**		**Diet**		**Interaction**	
	Control	Creosote	*F*	p	*F*	p	*F*	p
Food Intake (g) per gram Body Mass								
22°C	0.0709 ± 0.006	0.0574 ± 0.006	1.152	0.304	4.979	**0.046**	0.004	0.949
27°C	0.0642 ± 0.005	0.0514 ± 0.006						
Resin Intake (g) per gram Body Mass								
22°C	n/a	0.0023 ± 0.0002	7.463	**0.034**	n/a	n/a	n/a	n/a
27°C	n/a	0.0036 ± 0.0004						
Starting Body Mass (g)								
22°C	124.68 ± 8.02	123.96 ±10.98	0.085	0.775	0.027	0.872	0.009	0.928
27°C	128.56 ± 12.47	125.96 ± 8.08						
Ending Body Mass (g)								
22°C	123.38 ± 7.19	125.47 ± 10.15	0.058	0.814	0.022	0.884	0.132	0.723
27°C	129.27 ± 12.80	124.48 ± 7.91						
Liver Mass (g)								
22°C	3.6 ± 0.2	4.1 ± 0.5	1.559	0.236	0.25	0.626	0.877	0.367
27°C	3.5 ± 0.5	3.3 ± 0.4						
Liver Mass, relative to Body Mass (%)								
22°C	3.0	3.4	4.522	**0.055**	0.614	0.449	1.253	0.285
27°C	2.7	2.7						

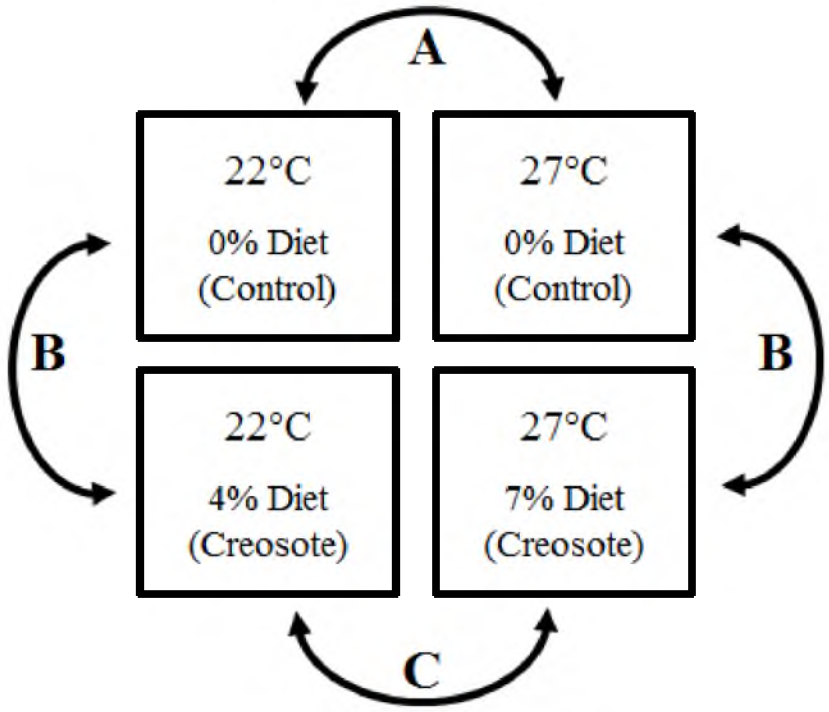

Figure 3.1: Diagram of four treatment groups in current study. Boxes represent experimental groups based on temperature and diet. Arrows and letters represent pair-wise comparisons made in GeneSifter to investigate differential gene expression between treatments. Comparison A represents an effect of temperature, and the two comparisons are labelled B because of their similar nature of distinguishing induction, just at two different temperatures.

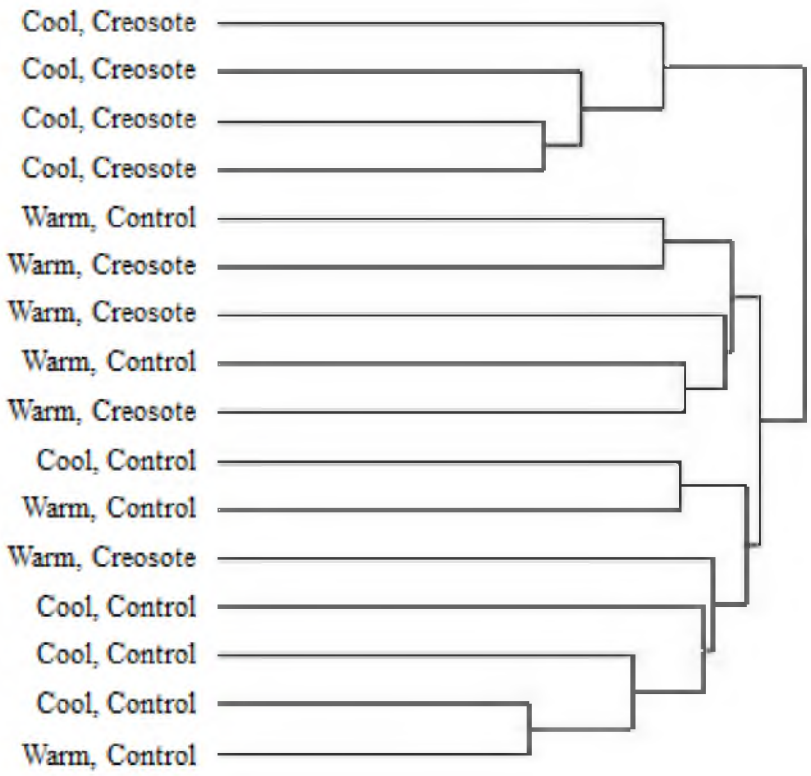

Figure 3.2: Cluster analysis of individual woodrat samples calculated from overall gene expression profiles using GeneSifter. Individuals are specified by temperature (Cool=22°C, Warm=27°C) and diet treatments (Control and Creosote).

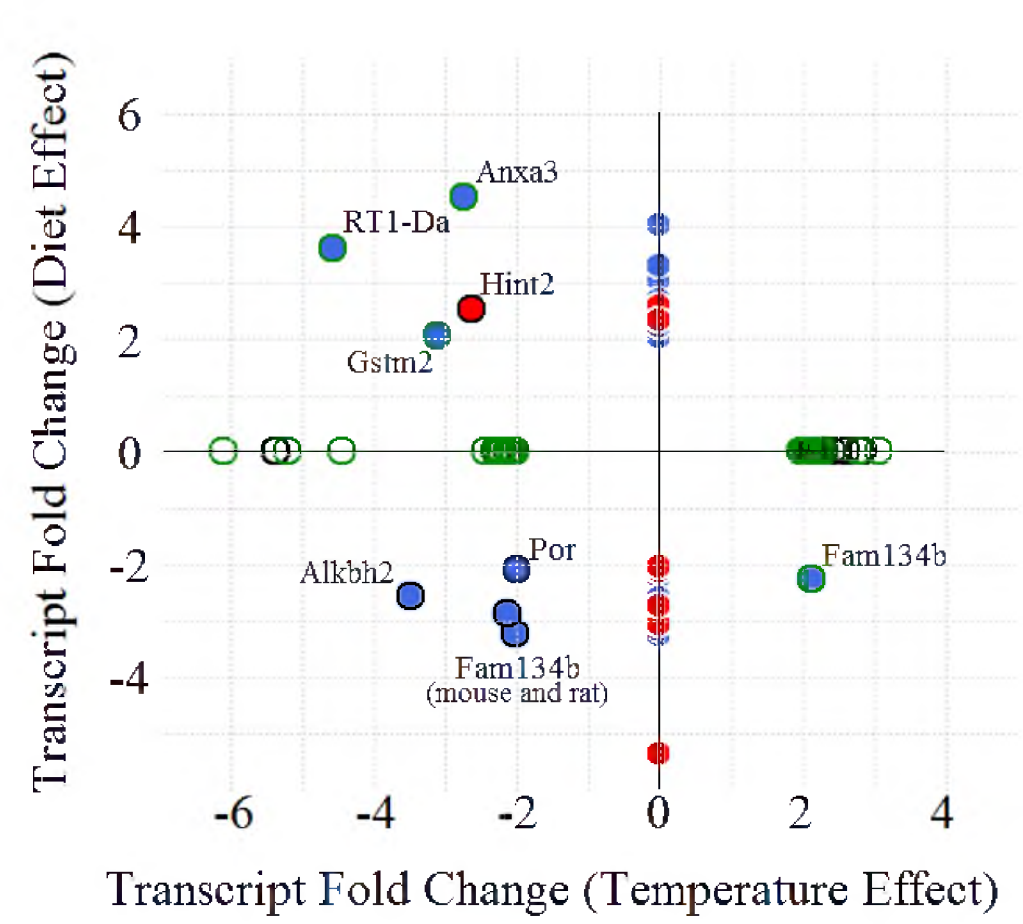

Figure 3.3: Differential gene expression due to dietary creosote and temperature in herbivorous woodrats. Scatterplot of individual transcripts appearing in more than one pair-wise comparison with gene label. The x-axis represents transcript fold changes due to temperature (positive numbers = up-regulated at 27°C; negative numbers = up-regulated at 22°C). Shape outlines indicate transcripts that are influenced by temperature while animals are ingesting a control diet (black outline) or creosote diet (green outline). The y-axis represents transcript fold changes due to diet (positive numbers = induced on creosote; negative numbers = repressed on creosote). Colors indicate transcripts that are influenced by dietary creosote at 27°C (red fill) or 22°C (blue fill).

Table 3.2: Number of transcripts with significantly different expression in a comparison of animals at 27°C and 22°C on a diet without creosote resin.

	Number of Transcripts	
On control diet, without creosote resin	All	Detox
Higher expression at 27°C	8	2
Higher expression at 22°C	15	4

Table 3.3: Transcripts differentially expressed at 27°C and 22°C while woodrats were ingesting diets without creosote resin. **Bolded** transcripts are associated with detoxification of plant secondary compounds.

Accession Number	Gene ID	Gene Description	Fold Change	p-value
(a) Up-regulated (N=8) at 27°C				
NM_001024349	Tnfrsf18	Tumor necrosis factor receptor superfamily, member 18	2.89	0.019359
NM_008458	Serpina3c	Serine (or cysteine) peptidase inhibitor, clade A, member 3C	2.86	0.027503
NM_001038588	**Prodh2**	**Proline dehydrogenase (oxidase) 2**	**2.63**	**0.027824**
NM_001107561	Scgb1c1	Secretoglobin, family 1C, member 1	2.44	0.01316
NM_153598	**Ugt2b34**	**UDP glucuronosyltransferase 2 family, polypeptide B34**	**2.31**	**0.000806**
NM_020275	Tnfrsf10b	Tumor necrosis factor receptor superfamily, member 10b	2.28	0.038127
NM_029068	Snx16	Sorting nexin 16, transcript variant 1	2.17	0.032457
NM_001079939	Lmf2	Lipase maturation factor 2	2.13	0.002198
(b) Up-regulated (N=15) at 22°C				
NM_001168541	Tsku	Tsukushi, transcript variant 1	5.43	0.001
NM_031768	Itgae	Integrin, alpha E	5.41	0.029398
NM_001126273	Alkbh2	alkB, alkylation repair homolog 2 (E. coli)	3.52	0.004777
NM_026871	Hint2	Histidine triad nucleotide binding protein 2	2.66	0.016189
NM_017300	Baat	Bile acid Coenzyme A: amino acid N-acyltransferase (glycine N-choloyltransferase)	2.29	0.013922
NM_001170477	Grtp1	Growth hormone regulated TBC protein 1, transcript variant 1	2.2	0.008883
NM_013872	Pmm1	Phosphomannomutase 1, transcript variant 1	2.15	0.004978

Table 3.3 continued

Accession Number	Gene ID	Gene Description	Fold Change	p-value
NM_025459	Fam134b	family with sequence similarity 134, member B, transcript variant 2	2.15	0.044535
NM_175507	Slc35g1	Solute carrier family 35, member G1	2.15	0.012776
NM_010726	**Phyh**	**Phytanoyl-CoA hydroxylase**	**2.14**	**0.044698**
NM_001159626	**Hagh**	**Hydroxyacyl glutathione hydrolase, transcript variant 2**	**2.08**	**0.004238**
NM_001159626	**Hagh**	**Hydroxyacyl glutathione hydrolase, transcript variant 2**	**2.05**	**0.003393**
NM_001034912	Fam134b	family with sequence similarity 134, member B	2.04	0.026345
NM_199372	Eif4a1	Eukaryotic translation initiation factor 4A1	2.04	0.008621
NM_008898	**Por**	**P450 (cytochrome) oxidoreductase**	**2.02**	**0.016539**

Table 3.4: KEGG pathways overrepresented at 27°C and 22°C in animals ingesting diets without creosote resin. "List" indicates the number of genes highly expressed within the pathway. "Gene Set" indicates the total number of genes in that pathway included on the array.

KEGG Pathway	List	Gene Set	z-score
(a) Pathways up-regulated at 27°C on control diet			
Cytokine-cytokine receptor interaction (signaling and interaction)	2	32	7.25
Drug metabolism - cytochrome P450 (xenobiotic biodegradation and metabolism)	1	66	2.25
Metabolism of xenobiotics by cytochrome P450 (xenobiotic biodegradation and metabolism)	1	54	2.56
Retinol metabolism (metabolism of cofactors and vitamins)	1	42	2.98
Drug metabolism - other enzymes (xenobiotic biodegradation and metabolism)	1	39	3.11
Arginine and proline metabolism (amino acid metabolism)	1	37	3.21
Apoptosis (cell growth and death)	1	27	3.84
Porphyrin and chlorophyll metabolism (metabolism of cofactors and vitamins)	1	26	3.92
Steroid hormone biosynthesis (lipid metabolism)	1	26	3.92
Starch and sucrose metabolism (carbohydrate metabolism)	1	23	4.2
Natural killer cell mediated cytotoxicity (immune system)	1	21	4.41
Pentose and glucuronate interconversions (carbohydrate metabolism)	1	21	4.41
Ascorbate and aldarate metabolism (carbohydrate metabolism)	1	19	4.66
Other types of O-glycan biosynthesis (glycan biosynthesis and metabolism)	1	15	5.28
(b) Pathways up-regulated at 22°C on control diet			
Peroxisome (transport and catabolism)	2	47	5.21
Regulation of actin cytoskeleton (cell motility)	1	54	2.21
Bile secretion (digestive system)	1	28	3.31
Amino sugar and nucleotide sugar metabolism (carbohydrate metabolism)	1	21	3.9
Fructose and mannose metabolism (Carbohydrate metabolism)	1	17	4.38
Biosynthesis of unsaturated fatty acids (lipid metabolism)	1	13	5.06

Table 3.4 continued

KEGG Pathway	List	Gene Set	z-score
Primary bile acid biosynthesis (lipid metabolism)	1	12	5.28
Taurine and hypotaurine metabolism (metabolism of other amino acids)	1	2	13.27

Table 3.5: Number of transcripts with significantly different expression in a comparison of animals ingesting diets containing 0% and 4% concentrations of creosote resin at 22°C.

	Number of Transcripts	
At 22°C	All	Detox
Induced expression on creosote	29	1
Repressed expression on creosote	20	2

Table 3.6: Number of transcripts with significantly different expression in a comparison of animals ingesting diets containing 0% and 7% concentrations of creosote resin at 27°C.

	Number of Transcripts	
At 27°C	All	Detox
Induced expression on creosote	5	0
Repressed expression on creosote	5	0

Table 3.7: KEGG pathways overrepresented at 22°C due to dietary creosote. "List" indicates the number of genes highly expressed within the pathway. "Gene Set" indicates the total number of genes in that pathway included on the array.

KEGG Pathway	List	Gene Set	z-score
(a) Pathways induced by creosote at 22°C			
Hematopoietic cell lineage (immune system)	3	10	10.1
Regulation of actin cytoskeleton (cell motility)	2	54	2.33
Phagosome (transport and catabolism)	2	47	2.59
Retinol metabolism (metabolism of cofactors and vitamins)	2	42	2.81
Osteoclast differentiation (development)	2	28	3.67
Antigen processing and presentation (immune system)	2	24	4.04
Amoebiasis (infectious parasitic)	2	20	4.5
Cell adhesion molecules (CAMs) (signaling and interaction)	2	16	5.12
Leishmaniasis (infectious parasitic)	2	14	5.52
Intestinal immune network for IgA production (immune system)	2	11	6.3
Type I diabetes mellitus (endocrine and metabolic disease)	2	9	7.02
Graft-versus-host disease (immune disease)	2	7	8.03
NOD-like receptor signaling pathway (immune system)	1	17	2.28
Arrhythmogenic right ventricular cardiomyopathy (ARVC) (cardiovascular disease)	1	16	2.37
Viral myocarditis (cardiovascular disease)	1	15	2.47
Hypertrophic cardiomyopathy (HCM) (cardiovascular disease)	1	13	2.71
Prion diseases (neurodegenerative diseases)	1	13	2.71
Dilated cardiomyopathy (cardiovascular disease)	1	11	3
Ether lipid metabolism (lipid metabolism)	1	11	3
Primary immunodeficiency (immune disease)	1	7	3.89
ECM-receptor interaction (signaling and interaction)	1	6	4.24
Allograft rejection (immune disease)	1	6	4.24
Asthma (immune disease)	1	6	4.24

Table 3.7 continued

KEGG Pathway	List	Gene Set	z-score
Autoimmune thyroid disease (immune disease)	1	6	4.24
African trypanosomiasis (infectious parasitic)	1	4	5.28
Malaria (infectious parasitic)	1	3	6.15
(b) Pathways repressed by creosote 22°C			
Complement and coagulation cascades (immune system)	1	36	2.86
mTOR signaling pathway (signal transduction)	1	22	3.8
Regulation of autophagy (transport and catabolism)	1	11	5.53
Fat digestion and absorption (digestive system)	1	10	5.82

Table 3.8: KEGG pathways overrepresented at 27°C due to dietary creosote. "List" indicates the number of genes highly expressed within the pathway. "Gene Set" indicates the total number of genes in that pathway included on the array.

KEGG Pathway	List	Gene Set	z-score
(a) Pathways induced by creosote at 27°C			
Retinol metabolism (metabolism of cofactors and vitamins)	2	42	6.28
Insulin signaling pathway (endocrine system)	1	51	2.65
Glycolysis / Gluconeogenesis (carbohydrate system)	1	36	3.26
Starch and sucrose metabolism (carbohydrate system)	1	23	4.2
Adipocytokine signaling pathway (endocrine system)	1	21	4.41
RIG-I-like receptor signaling pathway (immune system)	1	18	4.79
Carbohydrate digestion and absorption (digestive system)	1	10	6.54
Galactose metabolism (carbohydrate metabolism)	1	7	7.86
(b) Pathways repressed by creosote at 27°C			
Insulin signaling pathway (endocrine system)	2	51	5.65
PPAR signaling pathway (endocrine system)	1	37	3.21
Glycolysis / Gluconeogenesis (carbohydrate metabolism)	1	36	3.26
Starch and sucrose metabolism (carbohydrate metabolism)	1	23	4.2
TGF-beta signaling pathway (signal transduction)	1	21	4.41
Fructose and mannose metabolism (carbohydrate metabolism)	1	17	4.94
Pentose phosphate pathway (carbohydrate metabolism)	1	10	6.54

Table 3.9: Transcripts differentially expressed by dietary creosote resin at 22°C. **Bolded** transcripts are associated with detoxification of plant secondary compounds.

Accession Number	Gene ID	Gene Description	Fold Change	p-value
(a) Transcripts induced by creosote (n=29)				
NM_013470	Anxa3	Annexin A3	4.5	0.003822636
NM_001082548	Spint2	Serine protease inhibitor, Kunitz type 2, transcript variant 2	4.02	0.001440211
NM_001008847	RT1-Da	RT1 class II locus Da	3.59	0.013811806
NM_010831	Sik1	Salt inducible kinase 1	3.3	0.046958124
NM_008361	Il1b	Interleukin 1 beta	3.18	0.023476141
NM_001109011	Marco	Macrophage receptor with collagenous structure	3.04	0.034344896
NM_007811	Cyp26a1	Cytochrome P450, family 26, subfamily a, polypeptide 1	2.81	0.040471163
NM_001168633	Irs2	Insulin receptor substrate 2	2.79	0.021525404
NM_008538	Marcks	Myristoylated alanine rich protein kinase C substrate	2.79	0.01085828
NM_130408	Cyp26a1	Cytochrome P450, family 26, subfamily a, polypeptide 1 (rat)	2.69	0.035570097
NM_001009353	Pla2g7	Phospholipase A2 group VII (platelet-activating factor acetylhydrolase plasma)	2.68	0.014862302
NM_133416	Bcl2a1d	B-cell leukemia/lymphoma 2 related protein A1	2.67	0.031529123
NM_013566	Itgb7	Integrin, beta 7	2.51	0.024003587
NM_010693	Lck	Lymphocyte protein tyrosine kinase, transcript variant 2	2.48	0.001669367
NM_017196	Aif1	Allograft inflammatory factor 1	2.43	0.023769092
NM_010545	Cd74	CD74 antigen (invariant polypeptide of major histo-compatibility complex class II antigen-associated), transcript variant 2	2.42	0.016246453
NM_008788	Pcolce	Procollagen C-endopeptidase enhancer protein	2.26	0.025029801
NM_053018	Cd9	CD9 molecule	2.22	0.007975429

Table 3.9 continued

Accession Number	Gene ID	Gene Description	Fold Change	p-value
NM_021278	Tmsb4x	Thymosin, beta 4, X chromosome	2.22	0.029075018
NM_001161845	Sgk1	Serum/glucocorticoid regulated kinase 1, transcript variant 1	2.2	0.037447343
NM_019337	Rgs10	Regulator of G-protein signaling 10	2.19	0.005030167
NM_010745	Ly86	Lymphocyte antigen 86	2.13	0.047867885
NM_008026	Fli1	Friend leukemia integration 1	2.12	0.018219037
NM_007908	Eef2k	Eukaryotic elongation factor-2 kinase, transcript variant 1	2.1	0.035473214
NM_009254	Serpinb6a	Serine (or cysteine) peptidase inhibitor, clade B, member 6a, transcript variant 2	2.08	0.02877654
XM_573272	Hmgb2l1	PREDICTED: High mobility group box 2-like 1	2.05	0.006933749
NM_008183	**Gstm2**	**Glutathione S-transferase mu 2**	**2.04**	**0.043152157**
NM_011171	Procr	Protein C receptor, endothelial	2.04	0.034190521
NM_011480	Srebf1	Sterol regulatory element binding transcription factor 1	2.01	0.034487851
(b) Transcripts repressed by creosote (n=20)				
NM_022331	Herpud1	homocysteine-inducible endoplasmic reticulum stress-inducible ubiquitin-like domain member 1	3.28	0.004690184
NM_025459	Fam134b	family with sequence similarity 134 member B (mouse)	3.25	0.005002436
NM_001106610	Hdac11	histone deacetylase 11	2.96	0.001963518
NM_001034912	Fam134b	family with sequence similarity 134 member B (rat)	2.9	0.001476838
NM_007468	Apoa4	apolipoprotein A-IV	2.69	0.00393323
NM_001014058	Usp18	ubiquitin specific peptidase 18	2.61	0.012047458
NM_001126273	Alkbh2	alkB alkylation repair homolog 2, alpha-ketoglutarate-dependent dioxygenase	2.59	0.014164162
NM_177200	Svopl	SV2 related protein homolog (rat)-like	2.55	0.008425302

Table 3.9 continued

Accession Number	Gene ID	Gene Description	Fold Change	p-value
NM_144549	Trib1	tribbles homolog 1 (Drosophila)	2.53	0.006293534
NM_001108341	Ulk1	Unc-51 like autophagy activating kinase 1	2.46	0.000257355
NM_145076	Trim24	tripartite motif-containing 24	2.44	0.009648674
NR_033304	Lypd6	LY6/PLAUR domain containing 6, transcript variant 2	2.28	0.032679002
NM_028066	F11	coagulation factor XI	2.27	0.025944877
NM_177589	Ulk4	unc-51-like kinase 4	2.24	0.013478162
NM_012050	Omd	osteomodulin	2.21	0.034943672
NM_008898	**Por**	**P450 (cytochrome) oxidoreductase**	**2.1**	**0.00387607**
NM_053433	**Fmo3**	**flavin containing monooxygenase 3**	**2.08**	**0.003943949**
NM_027498	Sik3	SIK family kinase 3	2.07	0.000372976
NM_028116	Pygo1	pygopus 1	2.05	0.006980471
NM_001011873	Xkr9	X Kell blood group precursor related family member 9 homolog	2.01	0.016440614

Table 3.10: Gene ontology (GO) terms overrepresented in a comparison of woodrats at 22°C ingesting diets with (4%) and without (0%) dietary creosote. "List" indicates the number of genes highly expressed within the term. "Gene Set" indicates the total number of genes in that ontology included on the array. "-" indicates a z-score of less than 2.

Ontology	List	Gene Set	Induced by creosote		Repressed by creosote	
			List	z-score	List	z-score
regulation of biological process	29	1682	20	4.17	9	-
biological regulation	29	1797	20	3.86	9	-
response to stimulus	25	1241	16	3.84	9	-
regulation of cellular process	24	1561	17	3.26	7	-
multicellular organismal process	22	933	14	4.12	8	-
cellular response to stimulus	21	882	13	3.86	8	2.15
protein metabolic process	19	1037	9	-	10	2.71
regulation of metabolic process	19	1086	12	2.57	7	-
negative regulation of biological process	18	681	12	4.36	6	-
regulation of biological quality	17	540	11	4.7	6	2.35
signaling	17	764	11	3.39	6	-
macromolecule modification	16	651	7	-	9	3.72
cellular protein metabolic process	16	917	7	-	9	2.58
response to stress	16	525	10	4.2	6	2.42
signal transduction	16	689	10	3.23	6	-
protein modification process	15	625	7	-	8	3.23
negative regulation of cellular process	15	632	10	3.53	5	-
developmental process	15	804	11	3.2	4	-
multicellular organismal development	14	696	10	3.19	4	-
regulation of macromolecule metabolic process	14	904	10	2.29	4	-
regulation of primary metabolic process	14	937	10	2.16	4	-
regulation of multicellular organismal process	12	328	8	4.55	4	2.08
regulation of catalytic activity	12	356	8	4.26	4	-
regulation of molecular function	12	414	8	3.75	4	-
response to chemical stimulus	12	533	8	2.93	4	-

Table 3.10 continued

Ontology	List	Gene Set	Induced by creosote List	Induced by creosote z-score	Repressed by creosote List	Repressed by creosote z-score
system development	12	587	8	2.63	4	-
anatomical structure development	12	669	8	2.23	4	-
protein phosphorylation	11	229	6	4.12	5	3.91
positive regulation of cellular metabolic process	11	392	7	3.24	4	-
phosphorylation	11	316	6	3.15	5	3.02
positive regulation of metabolic process	11	413	7	3.08	4	-
phosphate metabolic process	11	388	6	2.58	5	2.49
phosphorus metabolic process	11	388	6	2.58	5	2.49
immune system process	10	265	9	6.15	1	-
cell communication	10	381	7	3.33	3	-
cell surface receptor linked signaling pathway	10	305	6	3.25	4	2.24
organ development	10	435	7	2.93	3	-
cell differentiation	10	460	7	2.76	3	-
cellular developmental process	10	473	7	2.68	3	-

Table 3.11: Transcripts differentially expressed by dietary creosote resin at 27°C.

Accession Number	Gene ID	Gene Description	Fold Change	p-value
(c) Induced at 27°C (N=5)				
NM_130408	Cyp26a1	Cytochrome P450, family 26, subfamily a, polypeptide 1 (rat)	2.57	0.016953
NM_026871	Hint2	Histidine triad nucleotide binding protein 2	2.51	0.00115
NM_007811	Cyp26a1	Cytochrome P450, family 26, subfamily a, polypeptide 1 (mus)	2.5	0.023996
NM_008061	G6pc	Glucose-6-phosphatase, catalytic	2.33	0.011019
NM_026301	Rnf125	Ring finger protein 125	2.32	0.025441
(d) Reduced at 27°C (N=5)				
NM_012543	Dbp	D site of albumin promoter (albumin D-box) binding protein	5.38	0.00069
NM_017098	Fabp6	fatty acid binding protein 6 ileal	3.08	0.019632
NM_053716	Fbp2	fructose-1,6-bisphosphatase 2	2.75	0.042054
NM_145572	Gys2	glycogen synthase 2	2.15	0.003046
NM_013058	Id3	inhibitor of DNA binding 3	2.05	0.049316

Table 3.12: Number of transcripts with significantly different expression in a comparison of animals at 27°C and 22°C while ingesting dietary creosote.

	Number of Transcripts	
On creosote diet	All	Detox
Higher expression at 27°C	15	7
Higher expression at 22°C	20	13

Table 3.13: Transcripts differentially expressed at 27°C and 22°C while woodrats were ingesting diets with differing concentrations of creosote resin (7% and 4%, respectively). **Bolded** transcripts are associated with detoxification of plant secondary compounds.

Accession Number	Gene ID	Gene Description	Fold Change	p-value	Detox Phase
(a) Up-regulated (N=15) at 27°C on creosote diet					
NM_010009	Cyp27b1	Cytochrome P450 family 27 subfamily b polypeptide 1	3.1	0.036488	
NM_008380	Inhba	Inhibin beta-A	2.81	0.019084	
NM_001161712	**Gcat**	**Glycine C-acetyltransferase (2-amino-3-ketobutyrate-coenzyme A ligase) transcript variant 2**	**2.33**	**0.014763**	**2**
NM_027430	Mpc2	Mitochondrial pyruvate carrier 2	2.32	0.003115	
NM_016668	**Bhmt**	**Betaine-homocysteine methyltransferase**	**2.27**	**0.004031**	**2**
NM_007820	**Cyp3a16**	**Cytochrome P450 family 3 subfamily a polypeptide 16**	**2.22**	**0.012303**	**1**
NM_019879	Suclg1	Succinate-CoA ligase GDP-forming alpha subunit	2.21	0.011975	
NM_028066	F11	Coagulation factor XI	2.15	0.000628	
NM_020565	**Sult3a1**	**Sulfotransferase family 3A member 1**	**2.14**	**0.005354**	**2**
NM_020565	**Sult3a1**	**Sulfotransferase family 3A member 1**	**2.12**	**0.003583**	**2**
NM_012520	**Cat**	**Catalase**	**2.09**	**0.000542**	**1**
NM_001130490	Lrp1	Low density lipoprotein-related protein 1	2.09	0.005086	
NM_007410	**Adh5**	**Alcohol dehydrogenase 5 (class III) chi polypeptide**	**2.08**	**0.014544**	**1**
NM_001039219	Gm6086	predicted gene 6086	2.05	0.01652	
NM_025291	Sra1	Steroid receptor RNA activator 1 transcript variant 1	2.01	0.001236	
(b) Up-regulated (N=20) at 22°C on creosote diet					
NM_026672	**Gstm7**	**Glutathione S-transferase mu 7**	**6.15**	**0.032319**	**1**

Table 3.13 continued

Accession Number	Gene ID	Gene Description	Fold Change	p-value	Detox Phase
NM_026672	**Gstm7**	**Glutathione S-transferase mu 7**	**5.25**	**0.031764**	**2**
NM_001008847	RT1-Da	RT1 class II locus Da mRNA	4.62	0.015847	
NM_153468	Gzma	Granzyme A	4.49	0.008612	
NM_008183	**Gstm2**	**Glutathione S-transferase mu 2**	**3.13**	**0.039236**	**2**
NM_013470	Anxa3	Annexin A3	2.77	0.0386	
NM_019291	Car2	Carbonic anhydrase II	2.46	0.004389	
NM_178847	Cyp27a1	Cytochrome P450 family 27 subfamily a polypeptide 1	2.32	0.038006	
NM_011921	**Aldh1a7**	**Aldehyde dehydrogenase family 1 subfamily A7**	**2.25**	**0.018235**	**1**
NM_172759	**Ces2e**	**Carboxylesterase 2E transcript variant 1**	**2.23**	**0.001379**	**1**
NM_172759	**Ces2e**	**Carboxylesterase 2E transcript variant 1**	**2.22**	**0.001043**	**1**
NM_013467	**Aldh1a1**	**Aldehyde dehydrogenase family 1 subfamily A1**	**2.13**	**0.010268**	**1**
NM_001190330	**Ces2a**	**Carboxylesterase 2A transcript variant 2**	**2.13**	**0.007461**	**1**
NM_053262	Hsd17b11	Hydroxysteroid (17-beta) dehydrogenase 11	2.13	0.004929	
NM_001191676	**Ugt2b10**	**UDP glucuronosyltransferase 2 family polypeptide B10**	**2.12**	**0.007714**	**2**
NM_001007602	**Gsto1**	**Glutathione S-transferase omega 1**	**2.11**	**0.024801**	**2**
NM_011921	**Aldh1a7**	**Aldehyde dehydrogenase family 1 subfamily A7**	**2.08**	**0.024642**	**1**
NM_001013084	**Akr1b10**	**Aldo-keto reductase family 1 member B10 (aldose reductase)**	**2.07**	**0.008095**	**1**
NM_001007699	Cept1	Choline/ethanolamine phosphotransferase 1	2.05	0.001054	
NM_001191676	**Ugt2b10**	**UDP glucuronosyltransferase 2 family polypeptide B10**	**2.04**	**0.007988**	**2**

Table 3.14: Gene ontology (GO) terms overrepresented in animals at 27°C and 22°C while ingesting diets with differing concentrations of creosote resin (7% and 4%, respectively). "List" indicates the number of genes highly expressed within the term. "Gene Set" indicates the total number of genes in that ontology included on the array. "-" indicates a z-score of less than 2.

Ontology	List	Gene Set	Up-regulated at 22°C		Up-regulated at 27°C	
			List	z-score	List	z-score
metabolic process	24	2711	13	2.54	11	-
small molecule metabolic process	11	782	6	2.46	5	-
oxidation-reduction process	10	375	5	3.64	5	3.88

Table 3.15: KEGG pathways overrepresented in animals at 27°C and 22°C while ingesting diets with differing concentrations of creosote resin (7% and 4%, respectively). "List" indicates the number of genes highly expressed within the pathway. "Gene Set" indicates the total number of genes in that pathway included on the array.

KEGG Pathway	List	Gene Set	z-score
(a) Pathways upregulated at 27°C on creosote diet			
Metabolic pathways	6	527	2.81
Drug metabolism - cytochrome P450	2	66	3.19
Metabolism of xenobiotics by cytochrome P450	2	54	3.62
Retinol metabolism	2	42	4.22
Fatty acid metabolism	1	37	2.07
Glycolysis / Gluconeogenesis	1	36	2.11
Complement and coagulation cascades	1	36	2.11
Cytokine-cytokine receptor interaction	1	32	2.28
Propanoate metabolism	1	29	2.43
Glycine, serine and threonine metabolism	1	29	2.43
Tryptophan metabolism	1	28	2.49
Steroid hormone biosynthesis	1	26	2.6
Cysteine and methionine metabolism	1	23	2.81
Tyrosine metabolism	1	22	2.89
TGF-beta signaling pathway	1	21	2.97
Amyotrophic lateral sclerosis (ALS)	1	18	3.25
Citrate cycle (TCA cycle)	1	17	3.36
Linoleic acid metabolism	1	17	3.36
Steroid biosynthesis	1	9	4.79
(b) Pathways upregulated at 22°C on creosote diet			
Drug metabolism - cytochrome P450	3	66	4.14
Metabolism of xenobiotics by cytochrome P450	3	54	4.69
Glutathione metabolism	3	36	5.96
Tryptophan metabolism	1	28	2.01
Bile secretion	1	28	2.01
Pyruvate metabolism	1	27	2.06
Toxoplasmosis	1	27	2.06
Antigen processing and presentation	1	24	2.23
Staphylococcus aureus infection	1	23	2.29
Glycerophospholipid metabolism	1	22	2.36
Systemic lupus erythematosus	1	22	2.36
Pentose and glucuronate interconversions	1	21	2.43
Histidine metabolism	1	20	2.51
Ascorbate and aldarate metabolism	1	19	2.59
beta-Alanine metabolism	1	17	2.78

Table 3.15 continued

KEGG Pathway	List	Gene Set	z-score
Cell adhesion molecules (CAMs)	1	16	2.88
Glycerolipid metabolism	1	16	2.88
Neuroactive ligand-receptor interaction	1	15	3
Pancreatic secretion	1	15	3
Viral myocarditis	1	15	3
Leishmaniasis	1	14	3.12
Gastric acid secretion	1	13	3.26
Nitrogen metabolism	1	13	3.26
Primary bile acid biosynthesis	1	12	3.42
Ether lipid metabolism	1	11	3.59
Intestinal immune network for IgA production	1	11	3.59
Hematopoietic cell lineage	1	10	3.79
Collecting duct acid secretion	1	9	4.02
Type I diabetes mellitus	1	9	4.02
Graft-versus-host disease	1	7	4.62
Allograft rejection	1	6	5.02
Asthma	1	6	5.02
Autoimmune thyroid disease	1	6	5.02

CHAPTER 4

AMBIENT TEMPERATURE INFLUENCES TOLERANCE TO PLANT SECONDARY COMPOUNDS IN A MAMMALIAN HERBIVORE

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Ambient temperature influences tolerance to plant secondary compounds in a mammalian herbivore

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Growing evidence suggests that plant secondary compounds (PSCs) ingested by mammals become more toxic at elevated ambient temperatures, a phenomenon known as temperature-dependent toxicity. We investigated temperature-dependent toxicity in the desert woodrat (*Neotoma lepida*), a herbivorous rodent that naturally encounters PSCs in creosote bush (*Larrea tridentata*), which is a major component of its diet. First, we determined the maximum dose of creosote resin ingested by woodrats at warm (28–29°C) or cool (21–22°C) temperatures. Second, we controlled the daily dose of creosote resin ingested at warm, cool and room (25°C) temperatures, and measured persistence in feeding trials. At the warm temperature, woodrats ingested significantly less creosote resin; their maximum dose was two-thirds that of animals at the cool temperature. Moreover, woodrats at warm and room temperatures could not persist on the same dose of creosote resin as woodrats at the cool temperature. Our findings demonstrate that warmer temperatures reduce PSC intake and tolerance in herbivorous rodents, highlighting the potentially adverse consequences of temperature-dependent toxicity. These results will advance the field of herbivore ecology and may hone predictions of mammalian responses to climate change.

1. Introduction

The ability of herbivores to tolerate plant toxins plays a crucial role in herbivore feeding behaviour and diet selection [1]. Plant secondary compounds (PSCs) are produced by plants as a defence against herbivory [2]. In turn, herbivores have evolved behavioural and physiological countermeasures to avoid, metabolize or tolerate PSCs [3–5]. The capacity of herbivores to consume PSCs has traditionally been predicted by the detoxification limitation hypothesis [1,6], which posits that all herbivores have upper limits for PSCs. These thresholds are largely influenced by intrinsic factors, such as hepatic biotransformation enzyme activity [7,8]. Laboratory studies have established that mammalian herbivores ranging from domesticated goats and sheep [9,10] to wild marsupials [11,12] and rodents [13,14] significantly reduce food intake to avoid overingestion of PSCs, thereby remaining below PSC threshold doses (i.e. the amount of PSCs willingly ingested by an herbivore [13]).

Extrinsic factors are rarely considered in detoxification limits of herbivores, yet ambient temperature could be greatly impacting PSC tolerance in mammalian herbivores. Evidence from pharmacological [15,16] and agricultural studies [17–19] suggests that chemical toxicity is temperature-dependent, whereby compounds became more toxic at warmer temperatures. The mechanism, referred to as temperature-dependent toxicity, is the likely result of reduced liver function through changes in toxin clearance time, enzymatic reactions or gene expression of crucial detoxification pathways [20,21]. In addition to artificial systems, there is evidence for temperature-dependent toxicity from an ecologically and evolutionarily relevant context. Data from two species of herbivorous woodrats (genus *Neotoma*) revealed that woodrats decreased food intake of toxic plants and demonstrated reduced liver function at warmer ambient temperatures [22–24]. The interaction between temperature and liver

function could have critical implications for mammalian herbivores that must balance PSC detoxification with thermoregulation, particularly in a warming environment.

Here, we expand upon previous studies of temperature-dependent toxicity by investigating the effect of temperature-dependent toxicity on PSC tolerance in the desert woodrat (*Neotoma lepida*). We chose this species because woodrats from the Mojave Desert can ingest large quantities of creosote bush (*Larrea tridentata*; up to 75% of diet [25,26]). The resin from creosote leaves contains a complex mixture of hundreds of PSCs with the most abundant component being nordihydroguaiaretic acid, a phenolic ligand and known feeding deterrent [27]. Desert woodrats can consume doses of these PSCs that cause kidney cysts and death in laboratory rodents [28,29].

The objective of this study was to determine the effect of ambient temperature on PSC tolerance, as defined by creosote resin intake, in desert woodrats. We hypothesized that woodrats at warmer temperatures would display a lower tolerance to PSCs than woodrats at cooler temperatures. We provided ecologically relevant PSCs from creosote resin in experimental diets to *N. lepida* housed at different temperatures. PSC tolerance was measured by determining the maximum dose of creosote resin at cool (22°C) and warm (28°C) temperatures. We predicted that animals at the warm temperature would have a lower maximum dose than animals at the cool temperature. In a second experiment, we controlled for the dose (g per day) of creosote resin ingested by animals and measured PSC tolerance at cool (21°C), warm (29°C) and room (25°C) temperatures. We predicted a gradient result, with animals at the warm temperature demonstrating low tolerances, animals at 25°C demonstrating intermediate tolerances, and animals at the cool temperature demonstrating high tolerances for creosote resin.

2. Material and methods

(a) Animal and plant collection

Woodrats and creosotes leaves were collected from the Mojave Desert in southwestern Utah (37°06′ N, 133°58′ W) in July 2011 and May 2012. Animals were transported to an animal facility at the University of Utah and maintained in the laboratory for three weeks before experiments. Plant material was stored at −20°C, and creosote resin was extracted from leaves as described previously. For further details, see electronic supplementary material.

(b) Temperature treatments

Animals were housed at cool (21–22°C), room (25°C) and warm (28–29°C) ambient temperatures during experiments. These temperatures regimes were chosen based upon previous work investigating temperature-dependent responses in *Neotoma* [22–24,30], the ecological relevance for Mojave Desert *N. lepida* [24] and the metabolic physiology of *N. lepida*. In endotherms, there is a range of intermediate ambient temperatures over which metabolic rate is at its lowest and where mammals can maintain thermal homeostasis without additional energy expenditure called the thermal neutral zone (TNZ) [31]. The TNZ for *N. lepida* ranges 25–34°C; thus, the warm (28–29°C) and room (25°C) treatments are within the TNZ of *N. lepida*, whereas the cool (21–22°C) treatment is just below the TNZ [24]. None of the ambient temperatures used herein are considered thermally stressful to *N. lepida*, and similar food intakes at 25°C and 29°C confirmed equivalent energetic costs at these temperatures (electronic supplementary material, figure S1). Temperatures were regulated with thermostats and space heaters (DeLonghi, USA), when necessary, and were measured every 10 min with HOBO data loggers.

(c) Maximum dose of creosote resin at two temperatures

The effect of temperature on the maximum dose of PSCs ingested by *N. lepida* was determined in a feeding trial by gradually increasing the concentration of creosote resin added to the diet. The maximum dose was defined as the greatest dose of creosote resin consumed by each animal within a 24 h period (g resin ingested per day) during the trial [13]. Woodrats ($n = 16$) were acclimated to cool or warm temperatures for 14–21 days before the trial was conducted. Woodrats were housed in metabolic cages (Lab Products Inc., USA), and were provided ad libitum food and water throughout the experiment, whereas the dietary concentration of creosote resin slowly increased from 0% to 12% over 21 days (electronic supplementary material, table S3). Total food, creosote resin and water intake, and body mass were recorded daily. Woodrats were removed from the experiment when they lost more than 10% of their starting body mass, because further loss could be lethal. Intake data were log + 1-transformed. Maximum dose and average intake values were analysed with one-way ANOVAs across temperatures.

(d) Controlled dose of creosote resin at three temperatures

We conducted a second feeding trial to control for ingested doses (g per day) of creosote resin to ensure all woodrats consumed the same daily amount of creosote resin. The effect of temperature on food intake, body mass maintenance and persistence in the trial was determined for woodrats acclimated to one of three temperatures (cool, warm and room; $n = 10$ per group) for 14–21 days. Woodrats entered a 10-day feeding trial and the amount of creosote resin ingested was controlled at a constant, daily dose of 0.36 g resin per day, which is below the maximum dose determined for warm and cool temperatures in the first experiment (electronic supplementary material, table S4). Woodrats were provided ad libitum food and water, and were housed in shoebox cages with feeder hoods (Lab Products Inc.) to permit accurate estimates of food intake. Body mass and intakes of food, creosote resin and water were measured daily. Woodrats were removed from the trial if they lost more than 10% of starting body mass, and the number of days the animals remained in the trial were measured as persistence. Intake data were log-transformed. Daily average intake data were analysed with one-way ANOVAs across temperature treatments with Tukey's honest significance difference post hoc tests. Persistence in the trial was analysed with Kaplan–Meier survival curves and pairwise comparisons with Bonferroni corrections were conducted across temperatures. Statistical analyses were performed in R (R Core Team).

3. Results

(a) Maximum dose of creosote resin

Ambient temperature significantly affected the maximum tolerated dose of creosote resin ingested by desert woodrats. Warm-acclimated (28°C) animals had a maximum dose that was only two-thirds that of cool animals (22°C; figure 1; $F_{1,14} = 9.1$, $p = 0.009$). Temperature also influenced food, and creosote resin intake averaged across all days. Woodrats at 22°C ingested on average 63% more food (figure 2*a*; $F_{1,14} = 60.1$, $p < 0.001$) and 29% more creosote resin (figure 2*b*; $F_{1,14} = 6.7$, $p = 0.021$) per day compared with animals at 28°C. Daily and cumulative

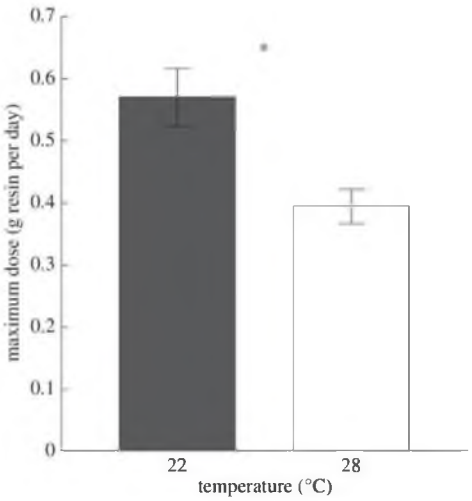

Figure 1. Maximum dose (mean ± s.e.) of creosote resin for woodrats acclimated to two ambient temperatures. Asterisk indicates significance.

intake values also reflected this pattern (electronic supplementary material, figures S2 and S3). Average daily water intake did not differ between temperatures (electronic supplementary material, table S5; $F_{1,14} = 0.8$, $p = 0.395$).

(b) Controlled dose of creosote resin

Creosote resin intake did not differ between temperature treatments during the second feeding trial (figure 3*a*; $F_{2,27} = 1.7$, $p = 0.206$); all woodrats ingested on average 0.36 g resin per day. There was no significant difference in food intake between warm (29°C) and room (25°C) temperatures (figure 3*b*; $p = 0.346$), but woodrats at the cool (21°C) temperature ingested three times more food than animals at other temperatures (figure 3*b*; $F_{2,27} = 87.7$, $p < 0.001$). Initial body mass ($F_{2,57} = 1.0$, $p = 0.37$) and water intake (electronic supplementary material, table S5; $F_{2,27} = 1.0$, $p = 0.37$) did not differ across temperatures.

There was a significant effect of temperature on woodrat persistence in the trial. Woodrats at 25°C and 29°C lost more body mass and were removed from the trial sooner compared with woodrats at 21°C (figure 4; $\chi^2_2 = 22.3$, $p < 0.001$). More than half of the woodrats at 29°C were unable to maintain body mass through day 5, and no animals remained by day 10. Similarly, more than half of the woodrats at 25°C dropped out of the trial by day 7 and only one animal remained by day 10. In contrast, all animals at 21°C persisted through the end of the trial. However, there was no difference in persistence between 25°C and 29°C (figure 4; $\chi^2_1 = 0.4$, $p > 0.5$).

4. Discussion

The interaction between ambient temperature and PSC tolerance is a relatively unexplored aspect of foraging behaviour, yet an interaction could have serious consequences for mammalian herbivores. Our results support the hypothesis that warm temperatures reduce ingestion of and tolerance to PSCs in the desert woodrat. The maximum dose of creosote resin was 30% lower with only a 6°C increase in temperature. Moreover, woodrats were unable to maintain body mass at temperatures

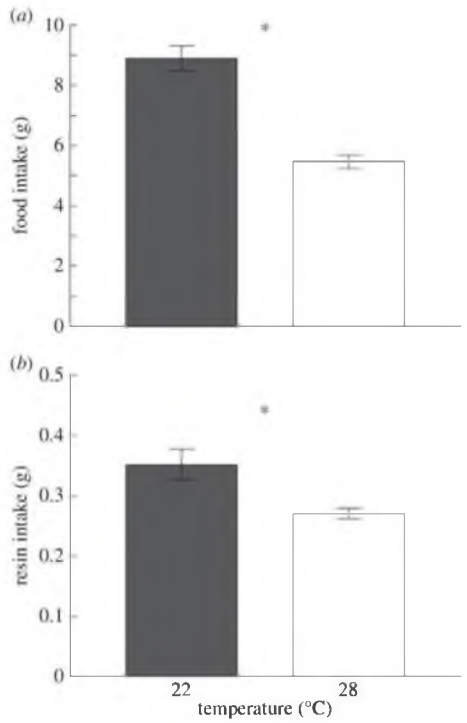

Figure 2. (*a*) Food and (*b*) creosote resin intake (mean ± s.e.) of woodrats at two temperatures averaged per individual ($n = 9$ at cool; $n = 7$ at warm) during 21 day trial. Asterisk indicates significance.

within the TNZ (25°C and 29°C), whereas woodrats at a cooler temperature just below the TNZ (21°C) were able to maintain mass when ingesting the same daily dose of creosote resin. These results provide further support for temperature-dependent toxicity and highlight the potentially negative impacts of this phenomenon on mammalian herbivores.

A temperature-mediated decrease in PSC tolerance could alter woodrat foraging behaviour in nature. As demonstrated here, a critical food source for the Mojave Desert population (i.e. creosote bush) is more potent at warmer temperatures. Woodrats will probably reduce intake of creosote bush by changing meal size and frequency of meals [32]. Diet switching could also be a viable strategy for woodrats to avoid PSCs when annual forbs and grasses are available [25,26], but few alternatives to creosote bush exist during much of the year in the Mojave Desert, particularly during the hottest months [33,34]. Additionally, changes in foraging behaviour could increase risk of predation. Given these constraints, woodrats may be forced to find new habitats to cope with decreased tolerance to creosote bush at warmer temperatures. Indeed, several species of small mammals including *Neotoma* have shifted their ranges to higher latitudes and elevations over the last 30–50 years, probably owing to climate change [35–37].

Contrary to our predictions, there was no difference in persistence on a controlled dose of creosote resin at 25°C versus 29°C, which are both within the TNZ. PSC tolerance in woodrats was predicted to decrease as temperatures rise across the TNZ owing to decreased liver function, resulting from a reduced ability to dissipate heat at warmer temperatures [21]. It is possible that the animals made physiological changes (e.g. shunting blood to the periphery) or behavioural changes

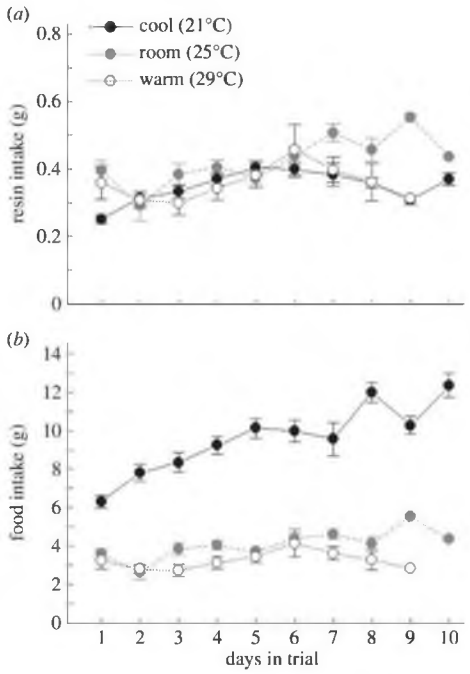

Figure 3. Daily (*a*) creosote resin and (*b*) food intake (mean ± s.e.) of woodrats at three temperatures (cool, black circles; room, grey circles; warm, white circles).

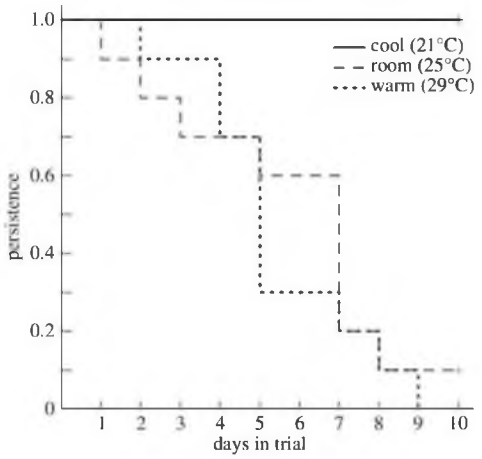

Figure 4. Proportion of woodrats remaining in the trial (persistence) while ingesting a controlled dose of creosote (0.36 g resin per day) at three temperatures (cool, solid line; room, grey dashed line; warm, dotted line).

in conductance to increase heat dissipation without reducing liver function. In addition, several liver enzymes are thought to play a role in metabolizing creosote resin ingested by animals held at room temperature [38]. We are currently investigating whether the expression of liver enzymes differs in woodrats exposed to different ambient temperatures.

Our results not only have immediate consequences for Mojave Desert woodrats, but are probably also relevant to other species. Mammalian herbivores must balance the physiological challenges of detoxifying PSCs in their diet with thermoregulation to maintain homeostasis; both these functions are critical roles played by the liver [39]. Temperature-dependent toxicity may also impact other endotherms facing similar physiological challenges, such as omnivorous European starlings [40]. Furthermore, these effects could be size-dependent; smaller mammals inherently have higher energetic costs than their larger counterparts, but can dissipate heat more readily [31]. A broader investigation of temperature-dependent toxicity across endotherms is a promising avenue for future research.

Temperature-dependent toxicity has implications for herbivorous mammals experiencing increased temperatures as a function of global climate change. The frequency and severity of extreme events (e.g. droughts and heat waves) are expected to increase, particularly in desert ecosystems [41,42]. Equally important is the documented rise in temperature minima, which can result in higher average surface temperatures [42]. Recent work also predicts declines in food quality and availability with climate change [37,43]. Temperature-dependent toxicity could amplify the impacts of these changes. Herbivorous mammals are crucial components of many ecosystems; thus, understanding their responses to climate change is imperative to developing conservation strategies.

In summary, our work provides novel evidence for temperature-dependent toxicity in an ecologically and evolutionarily relevant system, and supports the hypothesis that PSC tolerance decreases with ambient temperature. Our results help to fill knowledge gaps concerning the importance of extrinsic factors and their interactions with plant toxins in the current literature. A better understanding of these interactions will be likely to add a new dimension to the field of mammalian herbivore ecology.

Ethics. All procedures were approved by University of Utah Institutional Animal Care and Use Committee (12-12010) and Utah Division of Wildlife Resources (COR no. 1COLL5194).

Data accessibility. Data and R code: Dryad: http://dx.doi.org/10.5061/dryad.6q16d.

Authors' contributions. M.D.D. conceived of the study; P.K. and M.DD. designed the study; P.K. and N.D.M. participated in laboratory work and data analysis; P.K. wrote early drafts of the manuscript and all authors contributed substantially to revisions.

Competing interests. We have no competing interests.

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References

1. Freeland WJ, Janzen DH. 1974 Strategies in herbivory by mammals: the role of plant secondary compounds. *Am. Nat.* **108**, 269–289. (doi:10.2307/2459891)

2. Iason G. 2005 The role of plant secondary metabolites in mammalian herbivory: ecological

perspectives. *Proc. Nutr. Soc.* **64**, 123–131. (doi:10.1079/PNS2004415)
3. Fraenkel GS. 1959 The raison d'etre of secondary plant substances: these odd chemicals arose as a means of protecting plants from insects and now guide insects to food. *Science* **129**, 1466–1470.
4. Iason GR, Villalba JJ. 2006 Behavioral strategies of mammal herbivores against plant secondary metabolites: the avoidance–tolerance continuum. *J. Chem. Ecol.* **32**, 1115–1132. (doi:10.1007/s10886-006-9075-2)
5. Li Y, Feng Z, Swihart R, Bryant J, Huntly N. 2006 Modeling the impact of plant toxicity on plant–herbivore dynamics. *J. Dyn. Diff. Equ.* **18**, 1021–1042. (doi:10.1007/s10884-006-9029-y)
6. Karen JM, Ian RW, Rose LA, William JF. 2006 The detoxification limitation hypothesis: where did it come from and where is it going? *J. Chem. Ecol.* **32**, 1247–1266. (doi:10.1007/s10886-006-9082-3)
7. Dearing MD, Foley WJ, McLean S. 2005 The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. *Annu. Rev. Ecol. Evol. Syst.* **36**, 169–189.
8. McArthur C, Hagerman AE, Robbins CT. 1991 Physiological strategies of mammalian herbivores against plant defenses. In *Plant defenses against mammalian herbivory* (eds RT Palo, CT Robbins), pp. 103–114. Boston, MA: CRC Press.
9. Mote TE, Villalba JJ, Provenza FD. 2007 Relative availability of tannin-and terpene-containing foods affects food intake and preference by lambs. *J. Chem. Ecol.* **33**, 1197–1206. (doi:10.1007/s10886-007-9305-2)
10. Jansen DA, van Langevelde F, de Boer WF, Kirkman KP. 2007 Optimisation or satiation, testing diet selection rules in goats. *Small Ruminant Res.* **73**, 160–168. (doi:10.1016/j.smallrumres.2007.01.012)
11. Boyle R, McLean S. 2004 Constraint of feeding by chronic ingestion of 1,8-cineole in the brushtail possum (*Trichosurus vulpecula*). *J. Chem. Ecol.* **30**, 757–775. (doi:10.1023/B:JOEC.0000028430.92739.83)
12. DeGabriel JL, Moore BD, Shipley LA, Krockenberger AK, Wallis IR, Johnson CN, Foley WJ. 2009 Inter-population differences in the tolerance of a marsupial folivore to plant secondary metabolites. *Oecologia* **161**, 539–548. (doi:10.1007/s00442-009-1407-9)
13. Mangione AM, Dearing MD, Karasov WH. 2000 Interpopulation differences in tolerance to creosote bush resin in desert woodrats (*Neotoma lepida*). *Ecology* **81**, 2067–2076. (doi:10.1890/0012-9658(2000)081[2067:IDITTC]2.0.CO;2)
14. Skopec MM, Haley S, Dearing MD. 2007 Differential hepatic gene expression of a dietary specialist (*Neotoma stephensi*) and generalist (*Neotoma albigula*) in response to juniper (*Juniperus monosperma*) ingestion. *Comp. Biochem. Physiol. D, Genomics Proteomics* **2**, 34–43. (doi:10.1016/j.cbd.2006.11.001)
15. Keplinger ML, Lanier GE, Deichmann WB. 1959 Effects of environmental temperature on the acute toxicity of a number of compounds in rats. *Toxicology* **1**, 156–161. (doi:10.1016/0041-008x(59)90136-x)
16. Kaplanski J, Ben-Zvi Z. 1980 Effect of chronic heat exposure on *in vitro* drug metabolism in the rat. *Life Sci.* **26**, 639–642. (doi:10.1016/0024-3205(80)90240-4)
17. Aldrich CG, Paterson JA, Tate JL, Kerley MS. 1992 The effects of endophyte-infected tall fescue consumption on diet utilization and thermal regulation in cattle. *J. Anim. Sci.* **71**, 164–170.
18. Settivari R, Evans T, Eichen P, Rottinghaus G, Spiers D. 2008 Short-and long-term responses to fescue toxicosis at different ambient temperatures. *J. Therm. Biol.* **33**, 213–222. (doi:10.1016/j.jtherbio.2007.12.001)
19. Settivari R, Evans T, Yarru L, Eichen P, Sutovsky P, Rottinghaus G, Antoniou E, Spiers D. 2009 Effects of short-term heat stress on endophytic ergot alkaloid-induced alterations in rat hepatic gene expression. *J. Anim. Sci.* **87**, 3142–3155. (doi:10.2527/jas.2008-1684)
20. Gordon CJ, Johnstone A, Aydin C. 2014 Thermal stress and toxicity. *Compr. Physiol.* **4**, 995–1016. (doi:10.1002/cphy.c130046)
21. Dearing MD. 2013 Temperature-dependent toxicity in mammals with implications for herbivores: a review. *J. Comp. Physiol. B* **183**, 43–50. (doi:10.1007/s00360-012-0670-y)
22. Dearing MD, Forbey JS, McLister JD, Santos L. 2008 Ambient temperature influences diet selection and physiology of an herbivorous mammal, *Neotoma albigula*. *Physiol. Biochem. Zool.* **81**, 891–897. (doi:10.1086/588490)
23. McLister J, Sorensen J, Dearing M. 2004 Effects of consumption of juniper (*Juniperus monosperma*) on cost of thermoregulation in the woodrats *Neotoma albigula* and *Neotoma stephensi* at different acclimation temperatures. *Physiol. Biochem. Zool.* **77**, 305–312. (doi:10.1086/380211)
24. Kurnath P, Dearing MD. 2013 Warmer ambient temperatures depress liver function in a mammalian herbivore. *Biol. Lett.* **9**, 20130562. (doi:10.1098/rsbl.2013.0562)
25. Cameron GN, Rainey DG. 1972 Habitat utilization by *Neotoma lepida* in the Mohave desert. *J. Mammal* **53**, 251–266. (doi:10.2307/1379160)
26. Karasov WH. 1989 Nutritional bottleneck in a herbivore, the desert wood rat (*Neotoma lepida*). *Physiol. Zool.* **62**, 1351–1382.
27. Mabry TJ, Hunziker JH, Difeo Jr D. 1977 The natural products chemistry of Larrea. In *Creosote bush: biology and chemistry of Larrea in New World deserts* (eds TJ Mabry, JH Hunziker, DR DiFeo), pp. 115–134. New York, NY: Stroudsberg, Dowden, Hutchinson & Ross.
28. Goodman T, Grice H, Becking G, Salem F. 1970 A cystic nephropathy induced by nordihydroguaiaretic acid in the rat. Light and electron microscopic investigations. *Lab. Invest.* **23**, 93–107.
29. Rios JM, Mangione AM, Gianello JC. 2008 Effects of natural phenolic compounds from a desert dominant shrub *Larrea divaricata* Cav. on toxicity and survival in mice. *Revista chilena de historia natural* **81**, 293–302. (doi:10.4067/S0716-078X2008000200011)
30. Brown JH. 1968 Neotorna cinerea *and* N. albigula. Ann Arbor, MI: Museum of Zoology, University of Michigan.
31. Schmidt-Nielsen K. 1997 *Animal physiology: adaptation and environment*. Cambridge, UK: Cambridge University Press.
32. Torregrossa A-M, Dearing MD. 2009 Nutritional toxicology of mammals: regulated intake of plant secondary compounds. *Funct. Ecol.* **23**, 48–56. (doi:10.1111/j.1365-2435.2008.01523.x)
33. Hafner MS. 1977 Density and diversity in Mojave desert rodent and shrub communities. *J. Anim. Ecol.* **46**, 925–938. (doi:10.2307/3650)
34. Rundel PW, Gibson AC. 2005 *Ecological communities and processes in a Mojave Desert ecosystem*. Cambridge, UK: Cambridge University Press.
35. Smith FA, Betancourt JL. 2006 Predicting woodrat (Neotoma) responses to anthropogenic warming from studies of the palaeomidden record. *J. Biogeogr.* **33**, 2061–2076. (doi:10.1111/j.1365-2699.2006.01631.x)
36. Moritz C, Patton J, Conroy C, Parra J, White G, Beissinger S. 2008 Impact of a century of climate change on small-mammal communities in Yosemite National Park, USA. *Science* **322**, 261–264. (doi:10.1126/science.1163428)
37. Cahill A *et al.* 2013 How does climate change cause extinction? *Proc. R. Soc. B* **280**, 20121890. (doi:10.1098/rspb.2012.1890)
38. Malenke JR, Skopec MM, Dearing MD. 2014 Evidence for functional convergence in genes upregulated by herbivores ingesting plant secondary compounds. *BMC Ecol.* **14**, 23. (doi:10.1186/1472-6785-14-23)
39. Flanagan S, Ryan A, Gisolfi C, Moseley P. 1995 Tissue-specific HSP70 response in animals undergoing heat stress. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **268**, R28–R32.
40. Chatelain M, Halpin CG, Rowe C. 2013 Ambient temperature influences birds' decisions to eat toxic prey. *Anim. Behav.* **86**, 733–740. (doi:10.1016/j.anbehav.2013.07.007)
41. Parry ML, Canziani OF, Palutikof JP, Van der Linden PJ, Hanson CE. 2007 *Climate Change 2007: Impacts, Adaptation and Vulnerability*. Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge, UK: Cambridge University Press.
42. Field CB *et al.* 2014 Part A: global and sectoral aspects. In *Climate change 2014: impacts, adaptation, and vulnerability*. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, pp. 1–32. New York, NY: Cambridge University Press.
43. Zvereva EL, Kozlov MV. 2006 Consequences of simultaneous elevation of carbon dioxide and temperature for plant–herbivore interactions: a metaanalysis. *Glob. Change Biol.* **12**, 27–41. (doi:10.1111/j.1365-2486.2005.01086.x)

Table 4.S1: Summary of t-tests for body temperature and body mass in woodrats at two ambient temperatures (21°C and 27°C) feeding on a diet without creosote resin.

	t-test results		
	mean ± se	*p*	d.f.
Body temperature (°C)			
21°C	37.3 ± 0.1	0.772	1,9
27°C	37.2 ± 0.3		
Body mass (g)			
21°C	130.6 ± 12.1	0.915	1,9
27°C	132.6 ± 13.9		

Table 4.S2: Summary of t-tests for body temperature and body mass in woodrats at two ambient temperatures (21°C and 29°C) feeding on a diet with creosote resin.

	t-test results		
	mean ± se	*p*	d.f.
Body temperature (°C)			
21°C	37.7 ± 0.4	0.008	1,9
29°C	36.3 ± 0.1		
Body mass (g)			
21°C	129.4 ± 12.2	0.796	1,9
29°C	134.1 ± 13.1		

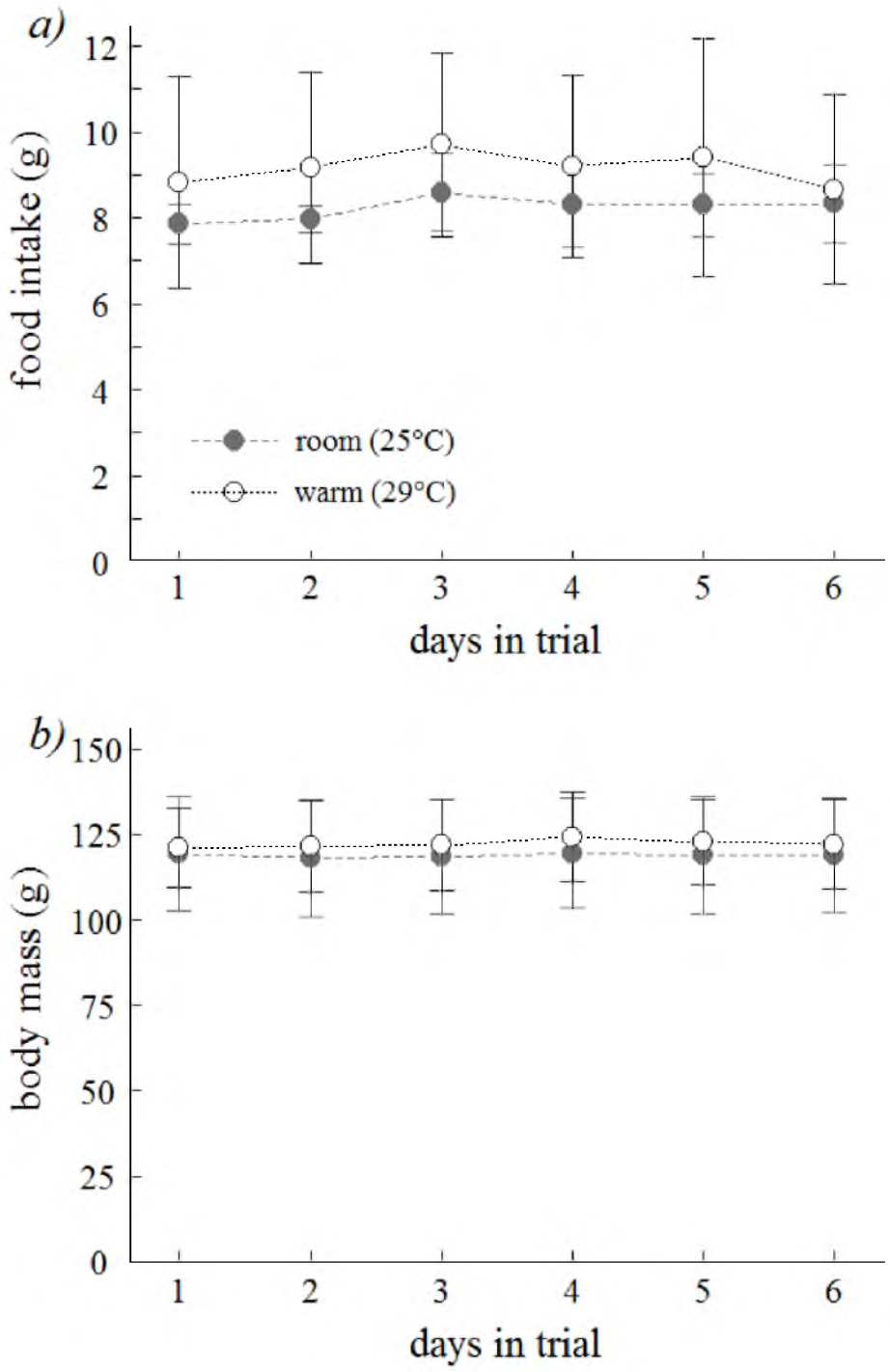

Figure 4.S1. Daily food intake (a) and body mass (b) of woodrats (mean ± se) during 6-day feeding trial. Different lines represent temperature groups: room (25°C, gray circles) and warm (29°C, open circles).

Table 4.S3: Experimental timeline to determine maximum dose of resin for woodrats at two temperatures (Cool and Warm), listing the days the trial that each group was presented a diet with a known concentration of creosote resin.

	Creosote resin presented in diet (%)						
	0	**2**	**4**	**6**	**8**	**10**	**12**
Group							
Cool (22°C)	Days 1-3	4-6	7-9	10-12	13-15	16-18	n/a
Warm (28°C)	Days 1-3	4-6	7-9	10-12	13-15	16-18	19-21

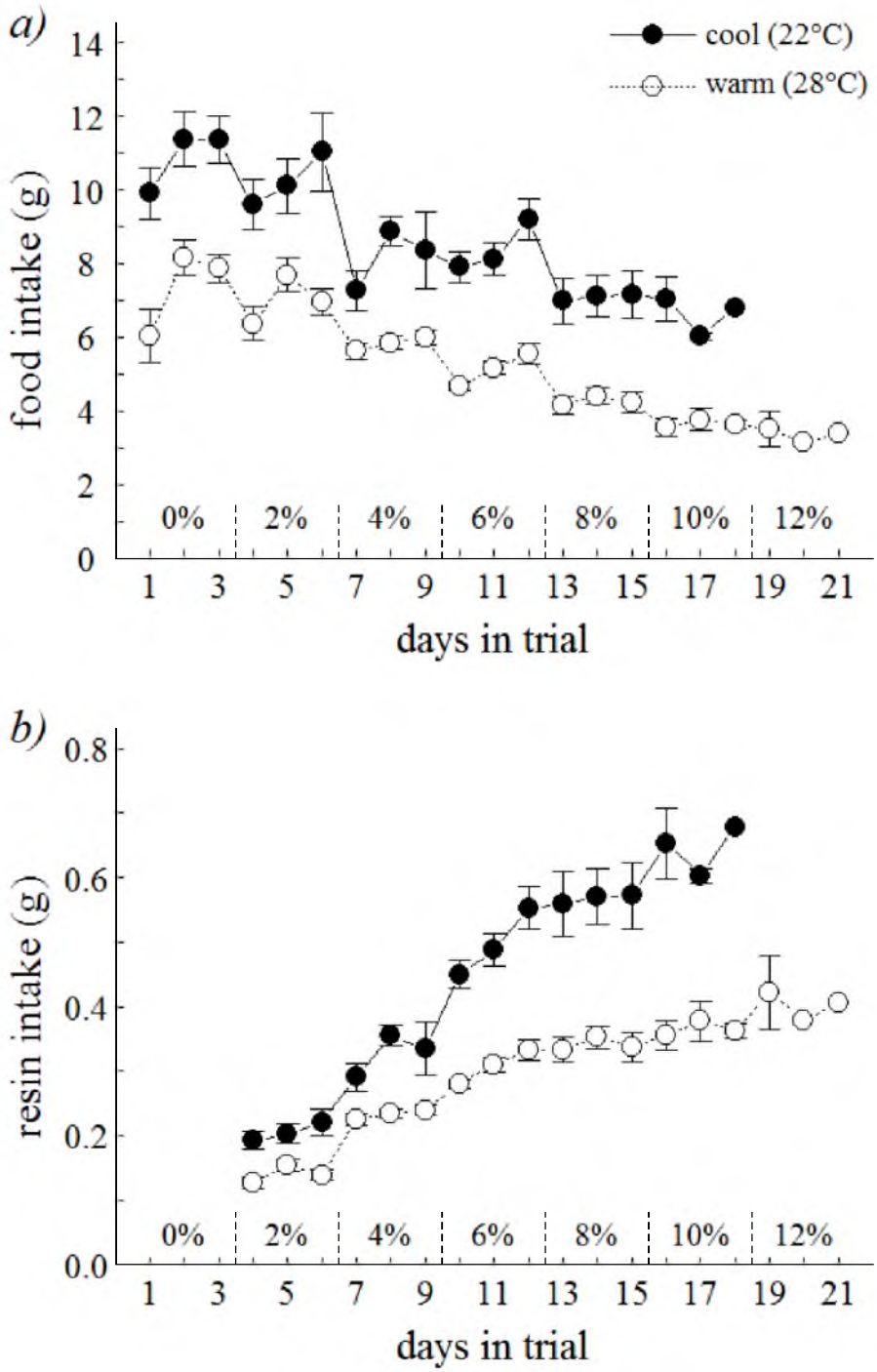

Figure 4.S2. Daily food (*a*) and creosote resin (*b*) intake (mean ± s.e.) of woodrats at two temperatures (cool=filled circles; warm=open circles). The dietary concentration of creosote resin (%) presented to woodrats is indicated along the x-axis.

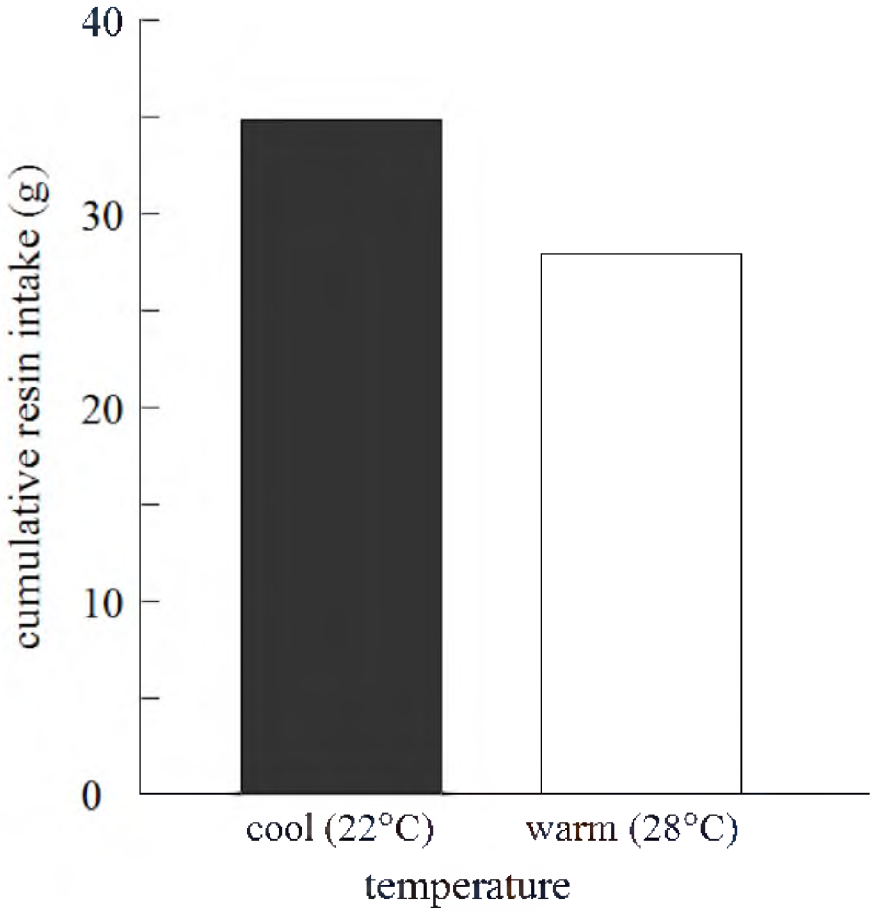

Figure 4.S3. Cumulative creosote resin intake of all woodrats at each of the two temperatures (cool = 34.78g; warm = 27.88g) at the end of a 21-day feeding trial.

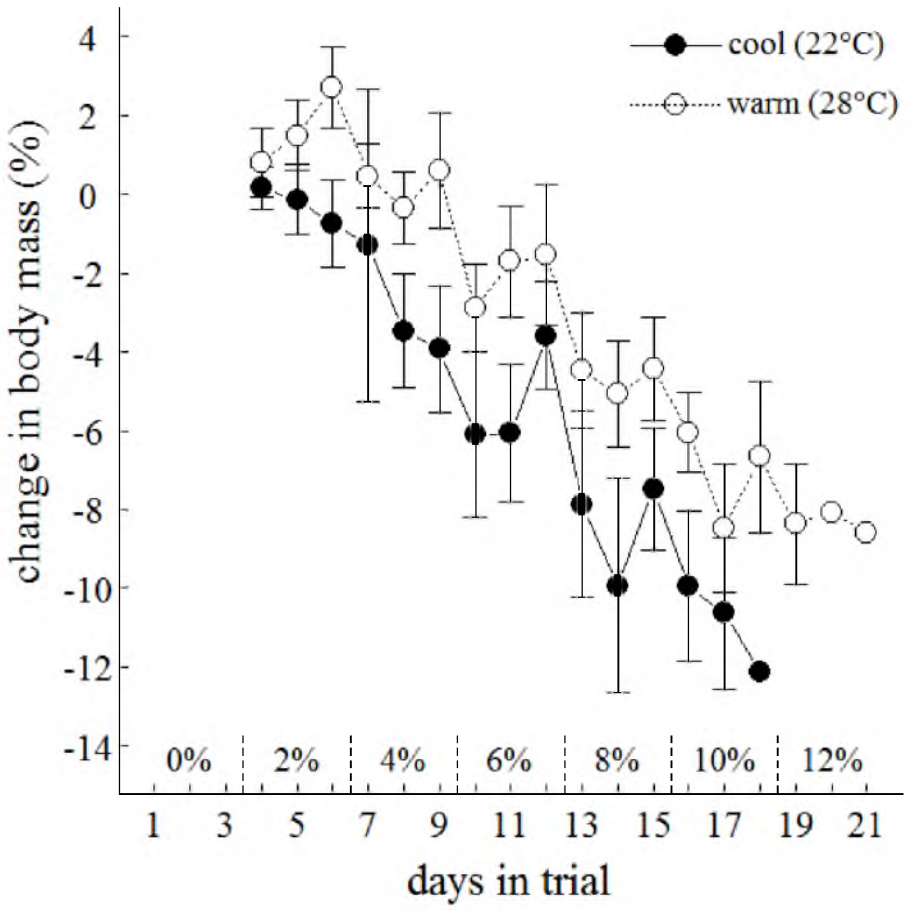

Figure 4.S4. Daily change in body mass (mean ± s.e.), represented as a percentage of starting body mass, of woodrats at two temperatures (cool=filled circles; warm=open circles). The dietary concentration of creosote resin (%) presented to woodrats is indicated along the x-axis. Individual animals were removed from the trial if percent change in body mass exceeded 10%

Table 4.S4: Experimental timeline to determine persistence of woodrats ingesting same daily dose of resin at three temperatures (Cool, Room, Warm), listing the number of days and the dietary concentration of creosote resin presented during those days

	Diet Acclimation		**Feeding Trial**
Number of Days	2	2	10
Group	*Creosote resin presented in diet (%)*		
Cool (22°C)	1	2	4
Room (25°C)	2.75	5.5	11
Warm (29°C)	2.75	5.5	11

Table 4.S5: Mean water intake by animals for both experiments at all temperature treatments. Intake values were averaged across all days in the trial per individual.

	Mean + SE
Maximum Dose (Expt 1)	
22°C	29.7 ± 0.1
28°C	25.7 ± 0.1
Controlled Dose (Expt 2)	
21°C	19.1 ± 1.2
25°C	28.5 ± 1.2
29°C	23.3 ± 1.3

CHAPTER 5

MAMMALIAN HERBIVORE USE OF MICROCLIMATES WHEN CHALLENGED BY DIETARY PLANT TOXINS

Abstract

Rising ambient temperatures pose a yet unrecognized threat to herbivorous mammals. Higher temperatures interact with mammalian physiology in such a way that foreign toxins become more potent, a phenomenon known as temperature-dependent toxicity (TDT). While the mechanism and effects of TDT have been previously investigated, it remains unknown whether mammalian herbivores will be able to mitigate the negative impacts of TDT. We predict that desert woodrats (*Neotoma lepida*) will use cooler microclimates to alleviate TDT and will have access to cooler microclimates in their natural habitat. The effects of dietary plant toxins on microclimate use and the influence of microclimate access on food intake and body mass maintenance were determined in a laboratory-based experiment. Additionally, temperature profiles of woodrat middens or nests in the Mojave Desert were collected over 17 months and compared to paired ambient temperatures. In the lab, woodrats spent equal time at cooler microclimates regardless of the presence or absence of dietary plant toxins. Access to cooler microclimates did not affect food intake but did result in significantly less body mass loss compared to woodrats without microclimate access. Summer temperature profiles

provided evidence that middens could provide cooler microclimates to woodrats in the Mojave Desert, particularly during the early hours of their inactive phase (i.e., morning). Taken together, these results suggest that cooler microclimates can mitigate TDT and exist in the natural habitat of desert woodrats in the Mojave Desert. Understanding how mammalian herbivores interact with their environment is imperative for management policies, especially during a period of climate change.

Introduction

Behavior plays a prominent role in mammalian thermoregulation, especially for species inhabiting warmer climates. As endotherms, mammals maintain a constant and high body temperature, and they have evolved numerous mechanisms to regulate body temperature at varying ambient temperatures (Degen 1997a, Schmidt-Nielsen 1997). Mammals use evaporative cooling to prevent overheating at hotter temperatures, but water quickly becomes the crucial and limiting resource, particularly for species living in dry environments (Degen 1997b). Therefore, mammals in arid habitats have adapted by physiologically reducing water loss and behaviorally avoiding extreme temperatures (Schmidt-Nielsen 1964, Schmidt-Nielsen 1997). This latter strategy is an important aspect of mammalian thermoregulation because it reduces water loss and is less energetically expensive compared to other physiological responses to heat stress, such as panting and saliva spreading (Schmidt-Nielsen 1964, Schmidt-Nielsen 1997).

The importance of microclimates and their role in providing cooler refugia has been well documented in the literature (Keppel et al. 2012, Potter et al. 2013, Storlie et al. 2014). By definition, refugia are locations within an environment that provide a stable

alternative or "safe-haven" from climate variability, and therefore, can be a means of survival in that environment (Mosblech et al. 2011, Ashcroft et al. 2012). For instance, accessibility to cooler microclimates greatly influenced population persistence of small mammals such as American pika (Varner and Dearing 2014) and Belding ground squirrels (Morelli et al. 2012). Also, mammals inhabiting arid ecosystems are dependent on underground nests and burrows, which provide cooler and more humid microclimates (Edelman 2011, Whittington-Jones et al. 2011).

Mammalian herbivores in particular must balance thermoregulatory challenges with the detoxification of plant defense compounds. Work from pharmacology and agriculture suggests that poisonous compounds and ambient temperature interact in such a way that toxins become more potent at warmer temperatures, a phenomenon known as temperature-dependent toxicity (TDT; Dearing 2013). Work conducted with laboratory rodents found that lethal doses for toxic compounds were lower at warmer temperatures (Keplinger et al. 1959), and that rodents sought out cooler temperatures when given a toxic agent (Gordon and Stead 1986a, b, Gordon et al. 2008). Likewise, animal scientists reported that higher temperatures made the negative effects of a fungal toxin found in grass worse for cattle and laboratory rats (Aldrich et al. 1992, Settivari et al. 2008, Spiers et al. 2008). This pattern of increased compound toxicity at higher ambient temperatures could have grave implications for wild mammalian herbivores that ingest plant toxins at every meal, especially in a time of climate change.

TDT has been investigated within an ecological and evolutionary context using woodrats or packrats (genus *Neotoma*). Woodrats are small, nocturnal rodents found across North America that can ingest a wide range of plants and subsequently are

exposed to myriad plant defense compounds (Cameron and Rainey 1972, Atsatt and Ingram 1983, Justice 1985, Dial 1988). Prior work has demonstrated that higher temperatures reduce liver function (Kurnath and Dearing 2013) as well as tolerance to plant toxins (Kurnath et al. 2016) in woodrats. These herbivores also have displayed a temperature-mediated change in diet selection to preferentially avoid plant toxins at higher temperatures (Dearing et al. 2008). Furthermore, woodrats construct middens or nests that can serve as microclimate refuges in environments as harsh as Death Valley (Brown 1968, Murray and Smith 2012). Here, we expand upon the investigation of TDT with a population of desert woodrats (*Neotoma lepida*) that inhabit the Mojave Desert and feed mainly on creosote bush (Larrea tridentata; Karasov 1989, Meyer and Karasov 1989).

In the current study, we set out to determine if woodrats could mitigate the negative effects of TDT by utilizing cooler microclimates. We conducted a laboratory-based feeding trial with desert woodrats to test the effects of ecologically relevant dietary toxins and microclimate access on animal behavior. Specifically, we predicted that (1) woodrats will use cooler microclimates when provided access and to a greater extent while ingesting creosote bush resin compared to a control diet lacking plant toxins, (2) woodrats with microclimate access will defend body mass loss to a greater degree than woodrats without microclimate access while fed dietary creosote resin, and (3) the ability of woodrats to maintain body mass is the result of being able to ingest more food when given microclimate access. Furthermore, we collected ambient and midden temperatures of desert woodrats in the Mojave Desert, testing the prediction that (4) woodrats have access to cooler microclimates in their desert habitat.

Materials and Methods

Animal collection: Woodrats (N=30) were collected from the Mojave Desert near Beaver Dam, Utah (37°06'N, 133°58'W) in May 2014. Creosote leaves were collected from several plants (>10) at the trapping site for the extraction of resin from leaves. Plant material was stored on dry ice in the field and at -20°C once at the University of Utah. Woodrats were transported to an animal facility at the University of Utah, and maintained in shoebox cages (48x27x20 cm) at room temperature (23-25°C) on a 12L:12D cycle. Woodrats were provided rabbit chow pellets (Teklad 2031 formula, Harlan Laboratories) and water *ad libitum* for at least three weeks before experiments. Experimental procedures were approved by the Institutional Animal Care and Use Committee (#12-12010).

Microclimate units: Specialized units were constructed to provide a choice of ambient temperatures (Figure 5.1). Two shoebox cages were connected via a small tube made of chicken wire (1-2in long, 3in diameter). Each cage contained bedding material (shavings and paper towel), water bottles and feeder hoods (Lab Products) to deter storage of food and allow continuous, unbiased access. Styrofoam walls lined with chicken wire controlled the space inhabited by the animals for location detection by similar-sized radio frequency identification devices.

Two microclimate treatments were used in the present study. One shoebox cage of the microclimate unit was maintained at 21°C (cool) and the second cage was maintained at 28°C (warm). To produce the microclimate, wall thermostats were set to 21°C and room temperatures were recorded every ten minutes during the trial (UA-001-08 HOBO pendant, Onset). Warm cages were heated to 28°C via terrarium heating pads

(Ultratherm Heat Pads, Bean Farm) secured to the bottom of selected cages with duct tape. Most units were assembled with one warm cage and one cool cage, thus providing access to a cooler microclimate. A portion of the units maintained both cages at 28°C, thus not providing access to a cooler microclimate. Data loggers (DS1920 iButton, Maxim Integrated) were secured on the bottom of each cage with duct tape during the trial. After the trial, data loggers were suspended one-half inch above the shavings for at least ten hours and temperature was recorded every 30 minutes.

Time spent by animals in each cage of their microclimate unit was recorded during the trial. Animals were outfitted with passive integrated transponder tags (PIT tags, TX1400ST, BioMark Inc.) prior to the trial, and location within the microclimate unit was monitored with handheld antennae connected to field computers (FS2001FT-ISO, BioMark Inc.). One antenna was located under each cage; thus, each microclimate unit included two antennae and two computers (Figure 5.1). Two Styrofoam pieces (1x1x12in) were placed between the antennae and cage bottoms to prevent interference from the heating pads.

Diet treatments: Animals were fed one of two dietary treatments: a control diet of ground rabbit chow (Harlan Laboratories) without creosote resin, and a creosote diet of ground rabbit chow amended with creosote bush resin (7%). This concentration represented natural amounts of resin found in Mojave Desert plants (Mabry et al. 1977). Briefly, creosote resin was extracted by soaking creosote leaves in acteone (1:6, wet leaf mass:volume solvent), after which the extract was vacuum filtered (Whatman filter paper, grade 1) and then evaporated under low pressure using a speedvac until a solid pellet remained. Resin was stored at -20°C prior to use. Diets were prepared as described

previously (Kurnath et al. 2016). Animals were fed a gradually increasing amount of resin over a six-day period to allow induction of biotransformation enzymes (Klaassen 2001). Animals in the creosote treatments received powdered rabbit chow with 0% resin for two days, followed by 2% resin for two days, and then 3.5% resin for two more days. Next, animals entered a five-day feeding trial during which they were presented 7% resin diets. Animals in the control diet treatment received diets containing 0% resin for the whole trial. A preliminary trial was conducted with animals on a higher dose of creosote resin (11%, maximum tolerable dose; Kurnath et al. 2016). However, these animals were unable to maintain body mass (i.e., >10% starting body mass), and a lower creosote concentration was used for the current experiment.

Feeding trial, data collection and analysis: This study contained three treatment groups, differentiated by dietary creosote resin and cooler microclimate access (Table 5.1). All animals were acclimated to 28°C for at least 14 days prior to the feeding trial. During temperature acclimation, animals were kept in home cages and provided rabbit chow pellets. Next, animals were moved into the specialized microclimate units and creosote resin was added gradually to ground rabbit chow over six days (described above). All animals then entered a five day feeding trial.

Body mass, food intake, creosote resin intake and water intake were recorded daily. Food intake was calculated by subtracting uneaten food from the amount of food offered 24 hours prior in each feeder hood, and both values from one unit were summed to calculate total food intake. Creosote resin intake was calculated as the product of total food intake per day multiplied by the proportion of creosote resin presented in the diet. Animals were removed from the trial if they lost more than 10% of their body mass after

being fed control diets for two nights in the microclimate units. Further loss of mass could be fatal in small rodents. Due to sample sizes and removal of animals from the trial, only data from Day 4 were used to compare food intake (g intake/g body mass) and change in body mass (%) between the treatments fed creosote diets with one-way ANOVAs. Cumulative creosote resin intake was also calculated across Days 1-4 and compared with a one-way ANOVA between groups fed creosote diets. The control group was excluded from this analysis because woodrats will decrease their overall food intake when plant toxins are added to their diet (Torregrossa and Dearing 2009).

Animal location was recorded once per minute during the woodrats' active phase (5PM – 8AM, dark cycle) during the trial. Data were transferred from the field computers to a desktop computer. Continuous time spent was summed for each cage, as defined by recordings within 60-80 seconds of the next recording. This 20-second time range accounted for potential lag time by the field computers. Next, the proportion of time spent in each cage of the microclimate unit was calculated between 7PM – 6AM for each animal. Average use of the cooler microclimate was calculated for every animal, and cooler microclimate use was compared across diet treatments using a one-way ANOVA. Animal location within the units was also recorded every 30 minutes during woodrats' inactive phase (i.e., 8AM – 5PM, light cycle). Due to experimenter activity and data collection at numerous points throughout the woodrats' inactive phase, time spent was analyzed from one day of the trial that best represented an uninterrupted three-hour time period (9AM – 12PM). Continuous time spent was calculated by merging the recordings from both cages of a microclimate unit into one file, and then summing continuous time between recordings for a single cage. Due to the rate of recordings set at

every 30 minutes, time spent for noncontinuous recordings was divided between both cages of the unit. The proportion of time spent in each cage of the microclimate unit was calculated over the three-hour inactive time phase for every animal, and averages were compared between dietary treatments using a one-way ANOVA. All statistical analyses were performed in R (R Core Team).

Collection of Field Temperatures: Temperature data loggers (UA-001-08 HOBO pendant) were deployed at ten middens or nests in the Mojave Desert from May 2014 – October 2015. Nests were found at the base of plants such as creosote bushes and Joshua trees, and chosen based upon occupancy when woodrats were collected in May 2014. Data loggers were deployed in a paired design at each nest. One logger was suspended 1-2M above ground, under a white plastic shield to prevent radiation from solar heat and within a 3M radius of the nest to record ambient temperatures (ambient location). A second logger was placed 8-12in within an active entry way (i.e., opening with fresh feces) of nests and secured to nearby branches (midden location). Temperatures were recorded once per hour. Data were offloaded from loggers in October 2014, May 2015, and October 2015. During each trip, data were recorded regarding nest occupancy (i.e., presence of fresh feces and plant clippings). Temperature data from June – August of each year were analyzed with HOBOware Pro software for three nests that were continuously occupied. Average and maximum monthly temperature values were calculated for locations at each nest (ambient and midden) and then averaged across all three nests. Monthly summer temperatures (June – August) for each year (2014 and 2015) were then compared with one-way repeated measures ANOVAs, using location (ambient and midden) as a main effect and month as a repeated measure.

Results

Microclimate Use: Woodrats spent a third of their time at the cooler temperature during their active phase (Figure 5.2a). Animals on the control diet spent on average 38.7% of their time at 21°C, and animals on the creosote diet spent an average of 32.9% of their time at 21°C. Dietary treatment did not significantly impact use of the cooler microclimate ($p=0.72$, $F_{1,10}=0.136$).

A similar pattern of microclimate use was found during the inactive phase of woodrats (Figure 5.2b). Animals presented the control diet spent about 43.6% of their time at 21°C, while animals fed the creosote diet spent 27.0% of their time at 21°C. Again, diet treatment had no effect on cooler microclimate use ($p=0.29$, $F_{1,10}=1.271$).

Food Intake and Body Mass: Woodrats fed diets containing creosote resin and provided access to a cooler microclimate did not ingest more food than animals without microclimate access ($p=0.62$, $F_{1,7}=0.263$; Figure 5.3a). Creosote resin intake ($p=0.65$, $F_{1,7}=0.227$) and cumulative resin intake were not significantly affected by access to a cooler microclimate ($p=0.46$, $F_{1,7}=0.602$).

Change in body mass of woodrats with microclimate access was significantly different from woodrats lacking microclimate access ($p=0.047$, $F_{1,7}=5.773$; Figure 5.3b). By Day 4, the difference in mean body mass loss was almost 3-fold between the two treatments presented creosote diets (microclimate = -1.15%; no microclimate = -3.85%).

Field Temperatures: During the summer months, the maximum midden temperature was consistently lower than the maximum ambient temperature (Figures 5.4a and 5.5a). Maximum midden temperatures were on average 7°C cooler than maximum ambient temperatures in both 2014 ($F_{1,2}=400$, $p=0.002$) and 2015 ($F_{1,2}=1067$, $p<0.001$). Average

summer temperatures did not significantly differ between the ambient and midden locations (Figures 5.4a and 5.5a). In 2014, average ambient and midden temperatures were 31.9°C and 31.2°C respectively ($F_{1,2}$=7.4, p=0.114), and 32.4°C and 31.7°C, respectively, in 2015 ($F_{1,2}$=7.8, p=0.108).

A closer inspection of daily temperature traces from three continuously occupied nests revealed distinctive patterns. In particular, midden temperatures were cooler than ambient temperatures between 7AM – 12PM in June 2014 (Figure 5.4b) and June 2015 (Figure 5.5b). These differences in temperature varied from as little as 4°C and up to 13°C. The daily range of midden temperatures for some nests was as little as 6°C, whereas the corresponding range of daily ambient temperature was 20°C (Figure 5.4b). However, for one nest in June 2014 (Figure 5.4b) and two nests in June 2015 (Figure 5.5b), midden temperatures rarely diverged more than 5°C from paired ambient temperatures outside of the 8AM – 12PM time frame.

Discussion

New evidence suggests that mammalian herbivores are adversely impacted by temperature-dependent toxicity (TDT) in such a way that plant defense compounds are physiologically more toxic at warmer temperatures. However, it remains unknown whether modifications in thermoregulatory behavior could mitigate the interactions between higher temperatures and ingested plant toxins. Here, we experimentally tested the hypothesis that access to cooler microclimates enhances the ability of herbivores feeding on toxic diets to survive in a warmer environment. We found that dietary creosote did not influence microclimate use by desert woodrats. Additionally, woodrats

without access to a cooler microclimate lost significantly more body mass than animals with microclimate access, even though food intake did not differ between these two treatments. The differences in body mass loss suggest that animals without the cooler microclimate would lose more body mass faster and potentially reach fatal levels of body mass sooner than animals with access to cooler microclimates. Lastly, temperature data collected from occupied nests in the Mojave Desert suggest that some middens provide thermal refugia to desert woodrats, particularly in the early hours of their inactive phase. Taken together, our results lend preliminary support to the hypothesis that access to cooler microclimates could offer desert woodrats a means to mitigate TDT.

In contrast with other studies on TDT, toxins did not influence the selection of microhabitats by the desert woodrat. We found that the time woodrats spent at 22°C did not differ between treatments with or without dietary creosote resin (Figure 5.2). Our results did not agree with prior studies that found laboratory rodents sought out cooler temperatures following an acute toxic challenge (Gordon and Stead 1986a, b, Gordon et al. 1988). However, these experiments recorded short-term thermal preferences of rats and mice housed in a thermal gradient (18-36°C) that were injected with high doses of foreign compounds. It is possible that the laboratory rodents were faced with a more severe physiological challenge which dictated a response. Alternatively, the creosote diets presented to woodrats in the current study contained ecologically relevant levels of plant toxins and thus were not overly challenging (Karasov 1989, Meyer and Karasov 1989).

While differences in the experimental protocols of previous experiments versus the one herein could explain these incongruent results, it is conceivable that animals in our

study were utilizing alternative thermoregulatory strategies that we did not measure. Mammals prevent overheating by first employing behavioral strategies, including changes in posture or actively seeking out cooler temperatures (Schmidt-Nielsen 1997). Next, fast-acting and often more energetically expensive physiological mechanisms are utilized, such as reducing heat production via changes in metabolism or regulating bodying temperature around a species-specific set point (Schmidt-Nielsen 1997). Additionally, rodents are known to spread saliva along appendages as a means of evaporative cooling (Hill and Wyse 1989). It is possible that woodrats in the current study used a combination of the aforementioned thermoregulatory strategies, and therefore had little need to utilize the cooler microclimate.

The potential benefits of having access to cooler microclimates were measured as body mass maintenance and food intake by desert woodrats. Animals with access to 22°C did not significantly increase their food intake compared to woodrats without access (Figure 5.3a). However, by Day 4 of the feeding trial, woodrats without access to 22°C lost substantially more body mass than animals with access, a difference that was almost three fold (Figure 5.3b). These results suggest that access to microclimates impacts energy efficiency, whereby animals lacking access to cooler temperatures spent more energy, and thus lose more body mass. Differences in activity level could explain the substantial body mass loss. Animals without access to 22°C could have spent more energy searching for alternative temperature environments or for other food sources; desert woodrats will reduce total creosote resin intake when provided a choice of diets (Appendix A). Woodrats lacking access to cooler temperatures also could have accrued a higher cost of thermoregulation that led to a greater loss in body mass. For instance,

higher activity levels could result in more muscle contractions, which is a cause for heat production in mammals (Schmidt-Nielsen 1997). Furthermore, woodrats housed at an ambient temperature within their thermal neutral zone (Kurnath and Dearing 2013), which is by definition not a thermally stressful state (Schmidt-Nielsen 1997), lost significantly more body mass than animals with microclimate access. Perhaps the term "thermal neutral" is a misnomer, since woodrats are experiencing an energy imbalance at 28°C without access to a cooler microclimate. In fact, laboratory rodents prefer to be at ambient temperatures just below their recorded thermal neutral zone (Gordon 1993). Recognizing these subtleties of mammalian thermoregulation is imperative to understanding the response of herbivores to TDT.

Regardless of the reason for the observed body mass loss, our results suggest that woodrats without access to a cooler microclimate would reach a 10% loss in body mass before woodrats with microclimate access. For instance, if a linear rate of change is assumed, then woodrats without microclimate access would reach an average of 10% body mass loss by Day 10 of the trial, whereas woodrats with access to a cooler microclimate would not reach 10% body mass loss until Day 34. The presence of a cooler microclimate most likely facilitated body mass maintenance and allowed woodrats to persist on a creosote diet longer compared to woodrats without microclimate access.

Temperature profiles from active nests in the Mojave Desert suggest that middens provide cooler refugia to desert woodrats. Midden temperatures buffered against ambient temperatures in the summer months by as much as 13°C, most often during the woodrats' inactive phase (8AM – 12PM; Figures 5.4b and 5.5b). This time frame could be ecologically crucial for woodrats, because these small herbivores not only feed on plant

material as they actively forage but also cache plant material in their middens to ingest later (Cameron and Rainey 1972, Dial 1988, Macêdo and Mares 1988, Karasov 1989). Thus, woodrats likely detoxify plant secondary compounds throughout the night as well as into the early hours of their inactive phase. Access to cooler temperatures as the ambient temperature rises quickly could provide woodrats the opportunity to mitigate TDT during the hottest months of the year. Differences in monthly maximum temperatures also provide support that middens act as refugia for desert woodrats in the Mojave Desert. Monthly maximum temperatures for middens and ambient temperatures were on average 7°C apart, which is similar to the difference between temperature treatments used in the current and previous experiments of TDT (Dearing et al. 2008, Kurnath and Dearing 2013, Kurnath et al. 2016). Lastly, summer midden temperatures occasionally remained below the documented lethal temperature for desert woodrats (41°C) during their inactive phase (Brown and Lee 1969).

Food availability in the Mojave Desert could be challenging for desert woodrats during the summer months. While alternative plants with fewer toxins are available during spring months (Cameron and Rainey 1972, Karasov 1989), creosote bush becomes the main food source for desert woodrats in the summer (Hafner 1977, Rundel and Gibson 2005). In fact, woodrats likely ingest concentrations of creosote resin comparable to the current study (7%) for most of the calendar year. Our results suggest that woodrats could maintain body mass on this concentration of creosote resin in the wild when cooler temperatures are available. Indeed, ambient temperatures recorded during the evening include the two temperature treatments used in our laboratory experiment (22°C and 28°C). While a nutritional bottleneck has previously been reported for desert woodrats of

the Mojave Desert as a result of a creosote diet (Karasov 1989), it is also possible that there is a summer bottleneck for desert woodrats due to TDT.

Global climate change could dramatically influence the impact of TDT on mammalian herbivores. In the face of rising ambient temperatures and more frequent and severe weather events (Parry et al. 2007, Field et al. 2014), herbivores will likely have to find new food sources or move to a different habitat in order to combat the adverse effects of TDT. Within the past 50 years, populations of small mammals, including *Neotoma* species, have shifted to higher latitudes and elevations (Moritz et al. 2008, Rowe et al. 2011). It is possible that TDT played a role in these range shifts as small mammals were pushed into historically unexperienced temperature regimes. As vital components of many ecosystems, understanding how mammalian herbivores may respond to climate change is essential for predicting repercussions and developing conservation strategies.

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References

Aldrich, C. G., J. A. Paterson, J. L. Tate, and M. S. Kerley. 1992. The effects of endophyte-infected tall fescue consumption on diet utilization and thermal regulation in cattle. Journal of Animal Acience 71:164-170.

Ashcroft, M. B., J. R. Gollan, D. I. Warton, and D. Ramp. 2012. A novel approach to quantify and locate potential microrefugia using topoclimate, climate stability, and isolation from the matrix. Global Change Biology 18:1866-1879.

Atsatt, P. R. and T. Ingram. 1983. Adaptation to oak and other fibrous, phenolic-rich foliage by a small mammal, *Neotoma fuscipes*. Oecologia 60:135-142.

Brown, J. H. 1968. Adaptation to Environmental Temperature in Two Species of Woodrats, Neotoma cinerea and Neotoma albigula. Miscellaneous Publications, Museum of Zoology, University of Michigan:1-48.

Brown, J. H. and A. K. Lee. 1969. Bergmann's Rule and Climatic Adaptation in Woodrats (*Neotoma*). Evolution 23:328-338.

Cameron, G. N. and D. G. Rainey. 1972. Habitat utilization by Neotoma lepida in the Mohave desert. Journal of Mammalogy 53:251-266.

Dearing, M. D. 2013. Temperature-dependent toxicity in mammals with implications for herbivores: a review. Journal of Comparative Physiology B 183:43-50.

Dearing, M. D., J. S. Forbey, J. D. McLister, and L. Santos. 2008. Ambient temperature influences diet selection and physiology of an herbivorous mammal, Neotoma albigula. Physiological and Biochemical Zoology 81:891-897.

Degen, A. A. 1997a. Heat Transfer and Body Temperature. Pages 27-60 Ecophysiology of Small Desert Mammals. Springer.

Degen, A. A. 1997b. Water Requirements and Water Balance. Pages 93-162 Ecophysiology of Small Desert Mammals. Springer.

Dial, K. P. 1988. Three sympatric species of *Neotoma*: dietary specialization and coexistence. Oecologia 76:531-537.

Edelman, A. J. 2011. Kangaroo Rats Remodel Burrows in Response to Seasonal Changes in Environmental Conditions. Ethology 117:430439.

Field, C. B., V.R. Barros, D.J. Dokken, K.J. Mach, M.D. Mastrandrea,, M. C. T.E. Bilir, K.L. Ebi, Y.O. Estrada, R.C. Genova, B. Girma, E.S. Kissel, A.N. Levy, S.

MacCracken,, and a. L. L. W. e. P.R. Mastrandrea, editors. 2014. Climate Change 2014: Impacts, Adapation, and Vulnerability. Cambridge University Press, New York, NY.

Gordon, C. 1993. Temperature regulation in laboratory rodents. Cambridge University Press, New York, NY.

Gordon, C. J., F. S. Mohler, W. P. Watkinson, and A. H. Rezvani. 1988. Temperature regulation in laboratory mammals following acute toxic insult. Toxicology 53:161-178.

Gordon, C. J., P. J. Spencer, J. Hotchkiss, D. B. Miller, P. M. Hinderliter, and J. Pauluhn. 2008. Thermoregulation and its influence on toxicity assessment. Toxicology 244:87-97.

Gordon, C. J. and A. G. Stead. 1986a. Effect of alcohol on behavioral and autonomic thermoregulation in mice. Alcohol 3:339-343.

Gordon, C. J. and A. G. Stead. 1986b. Effect of nickel and cadmium chloride on autonomic and behavioral thermoregulation in mice. Neurotoxicology 7:97-106.

Hafner, M. S. 1977. Density and Diversity in Mojave Desert Rodent and Shrub Communities. Journal of Animal Ecology 46:925-938.

Hill, R. W. and G. A. Wyse. 1989. Animal physiology. Harper & Row New York.

Justice, K. E. 1985. Oxalate digestibility in *Neotoma albigula* and *Neotoma mexicana.* Oecologia 67:231-234.

Karasov, W. H. 1989. Nutritional bottleneck in a herbivore, the desert wood rat (*Neotoma lepida*). Physiological Zoology 62:1351-1382.

Keplinger, M. L., G. E. Lanier, and W. B. Deichmann. 1959. Effects of environmental temperature on the acute toxicity of a number of compounds in rats. Toxicology 1:156-161.

Keppel, G., K. P. Van Niel, G. W. Wardell-Johnson, C. J. Yates, M. Byrne, L. Mucina, A. G. Schut, S. D. Hopper, and S. E. Franklin. 2012. Refugia: identifying and understanding safe havens for biodiversity under climate change. Global Ecology and Biogeography 21:393-404.

Klaassen, C. 2001. Casarett and Doull's Toxicology: The Basic Science of Poisons. McGraw Hill, New York.

Kurnath, P. and M. D. Dearing. 2013. Warmer ambient temperatures depress liver function in a mammalian herbivore. Biology Letters 9.

Kurnath, P., N. D. Merz, and M. D. Dearing. 2016. Ambient temperatures affect tolerance to plant secondary compounds in a mammalian herbivore. Proceedings of the Royal Society B 283.

Mabry, T. J., D. R. DiFeo, M. Sakakibara, C. Bohnstedt Jr, and D. Seigler. 1977. The natural products chemistry of Larrea. Pages 115-134 *in* T. J. Mabry, J. H. Hunziker, and D. R. DiFeo, editors. Creosote bush: biology and chemistry of Larrea in New World deserts. Stroudsburg, Dowden, Hutchinson & Ross, New York, NY.

Macêdo, R. H. and M. A. Mares. 1988. *Neotoma albigula*. Mammalian species 310:1-7.

Meyer, M. W. and W. H. Karasov. 1989. Antiherbivore chemistry of Larrea tridentata: effects on woodrat (*Neotoma lepida*) feeding and nutrition. Ecology 70:953-961.

Morelli, T. L., A. B. Smith, C. R. Kastely, I. Mastroserio, C. Moritz, and S. R. Beissinger. 2012. Anthropogenic refugia ameliorate the severe climate-related decline of a montane mammal along its trailing edge. Proceeding of Royal Society B 279:4279-4286.

Moritz, C., J. Patton, C. Conroy, J. Parra, G. White, and S. Beissinger. 2008. Impact of a century of climate change on small-mammal communities in Yosemite National Park, USA. Science 322:261-264.

Mosblech, N. A. S., M. B. Bush, and R. van Woesik. 2011. On metapopulations and microrefugia: palaeoecological insights. Journal of Biogeography 38:419-429.

Murray, I. W. and F. A. Smith. 2012. Estimating the influence of the thermal environment on activity patterns of the desert woodrat (Neotoma lepida) using temperature chronologies. Canadian Journal of Zoology 90:1171-1180.

Parry, M. L., O. F. Canziani, J. P. Palutikof, P. J. Van der Linden, and C. E. Hanson, editors. 2007. Climate Change 2007: Impacts, Adaptation and Vulnerability. Contribution of working group II to the fourth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge, UK.

Potter, K., H. Arthur Woods, and S. Pincebourde. 2013. Microclimatic challenges in global change biology. Global Change Biology 19:2932-2939.

Rowe, R., R. Terry, and E. Rickart. 2011. Environmental change and declining resource availability for small-mammal communities in the Great Basin. Ecology 92:1366-1375.

Rundel, P. W. and A. C. Gibson. 2005. Ecological communities and processes in a Mojave Desert ecosystem. Cambridge University Press.

Schmidt-Nielsen, K. 1964. Desert animals: physiological problems of heat and water. Clarendon Press.

Schmidt-Nielsen, K. 1997. Animal physiology: adaptation and environment. Cambridge University Press.

Settivari, R., T. Evans, P. Eichen, G. Rottinghaus, and D. Spiers. 2008. Short-and long-

term responses to fescue toxicosis at different ambient temperatures. Journal of Thermal Biology 33:213-222.

Spiers, D. E., T. J. Evans, and G. E. Rottinghaus. 2008. Interaction between thermal stress and fescue toxicosis: Animal models and new perspectives.*in* C. P. W. C.A. Roberts, D.E. Spiers, editor. Neotyphodium in Cool-Season Grasses. Blackwell Publishing Ltd, Oxford, UK.

Storlie, C., A. Merino-Viteri, B. Phillips, J. VanDerWal, J. Welbergen, and S. Williams. 2014. Stepping inside the niche: microclimate data are critical for accurate assessment of species' vulnerability to climate change. Biology Letters 10:1426158112.

Torregrossa, A.-M. and M. D. Dearing. 2009. Nutritional toxicology of mammals: regulated intake of plant secondary compounds. Functional Ecology 23:48-56.

Varner, J. and M. D. Dearing. 2014. The importance of biologically relevant microclimates in habitat suitability assessments. PLoS One 9:e104648.

Whittington-Jones, G. M., R. T. F. Bernard, and D. M. Parker. 2011. Aardvark Burrows: A Potential Resource for Animals in Arid and Semi-Arid Environments. African Zoology 49:362-370.

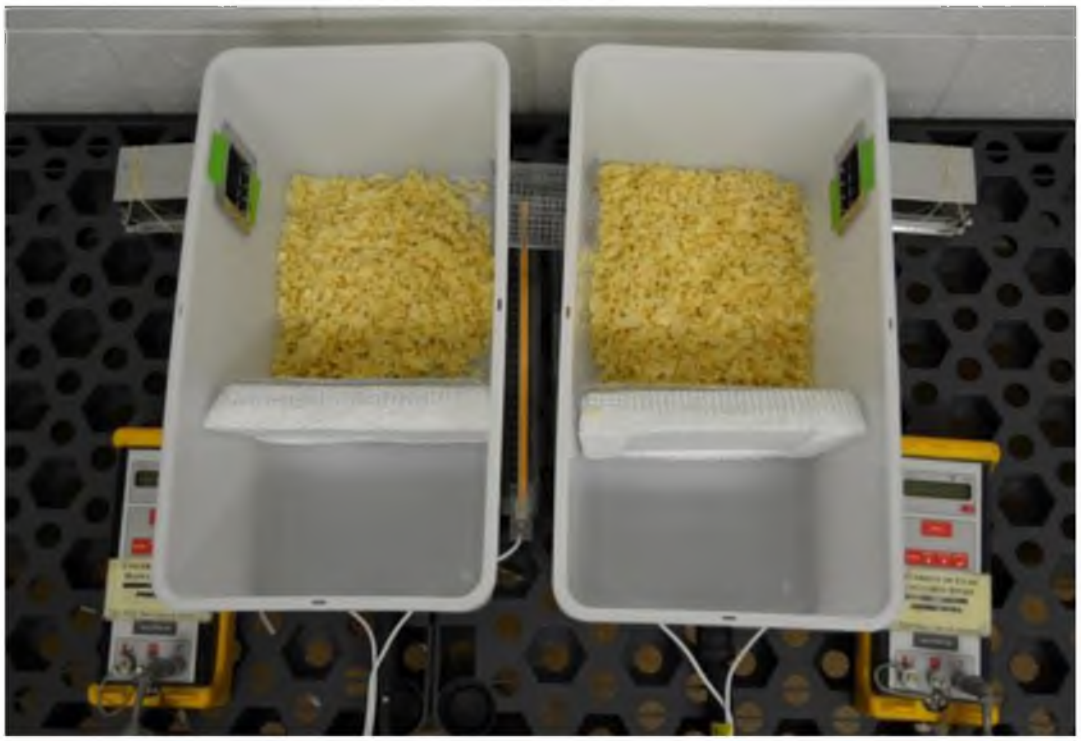

Figure 5.1: Image of microclimate unit constructed for the current study to allow temperature control of each cage within the unit. Cage tops have been removed to allow better visualization of the unit. Photograph by Mandy Giles.

Table 5.1: Definition of treatment groups in the current study, distinguished by access to a cooler microclimate (yes or no) or concentration of dietary creosote presented in the diet (0% or 7%).

	Dietary Creosote Concentration	**Microclimate Access**	**Sample Size**
Control	0%	Yes	7
Creosote	7%	Yes	5
Creosote without access	7%	No	4

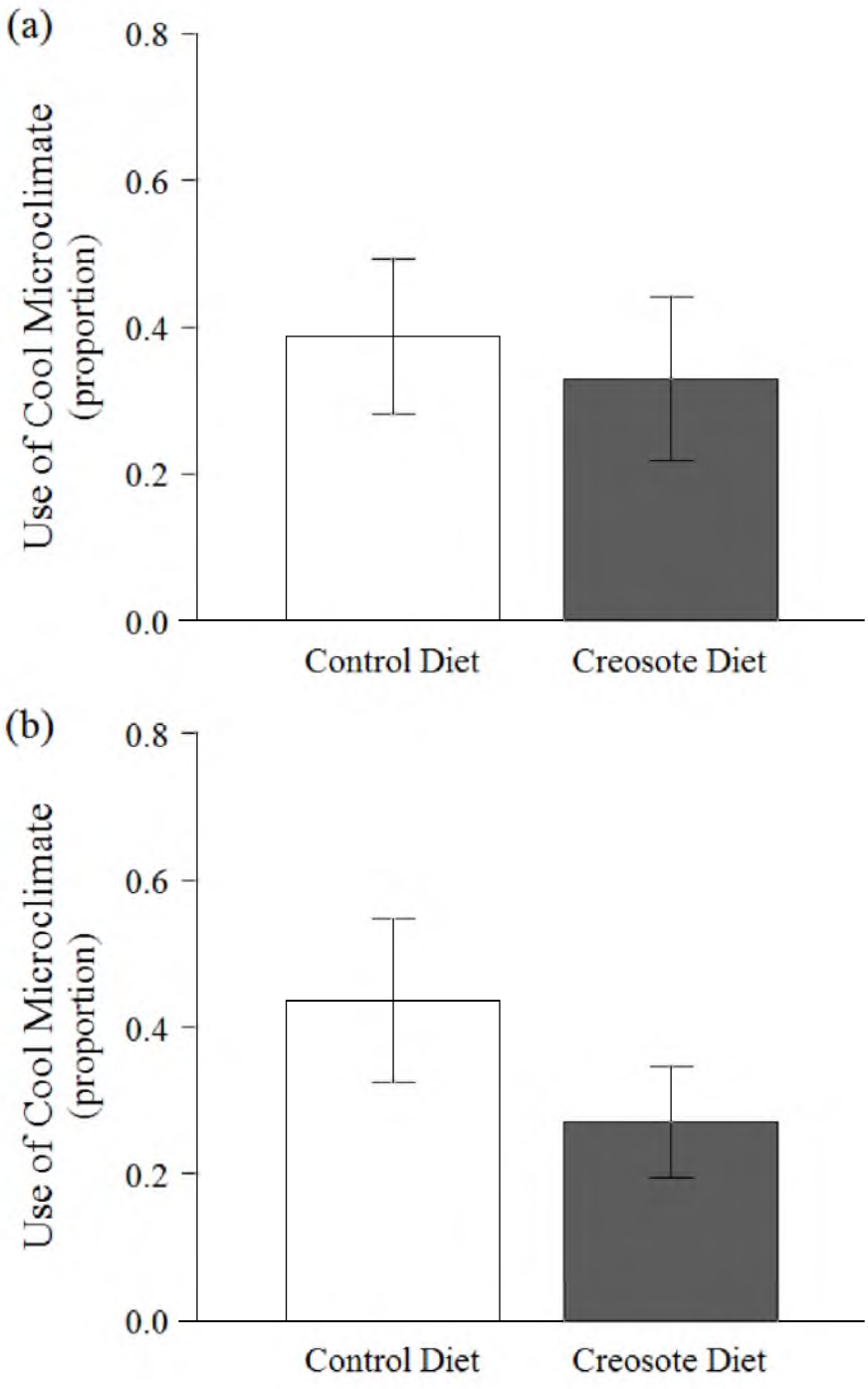

Figure 5.2: Proportion of time spent at the cool microclimate (mean ± SE) by woodrats during (a) active and (b) inactive phases. Time spent during active phases (7PM-6AM) was averaged per individual across Days 1-4 in the feeding trial. Time spent during inactive phases (9AM-12PM) was determined per individual only during Day 4 of the feeding trial.

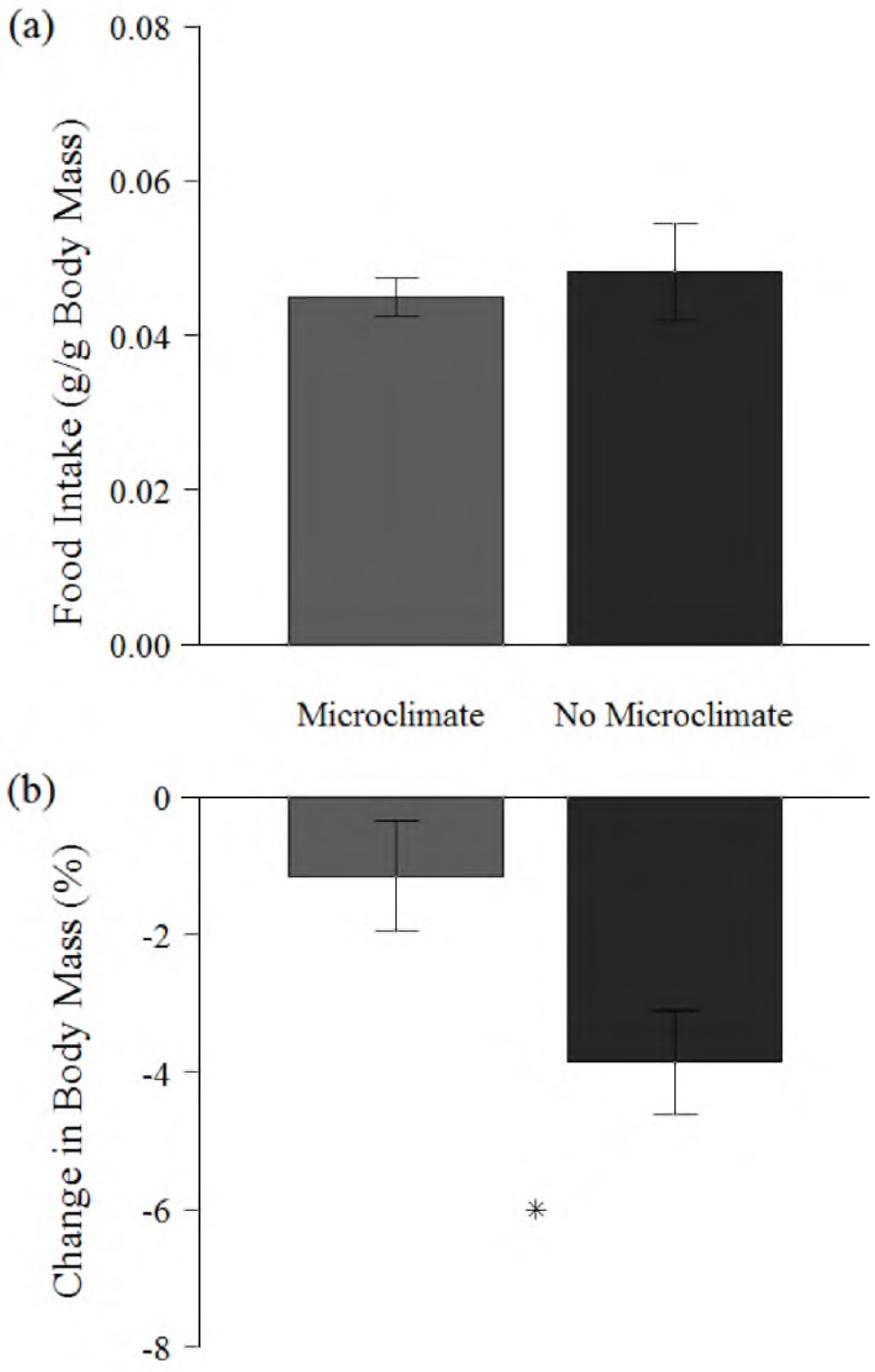

Figure 5.3: (a) Food intake corrected for body mass (mean ± SE) and (b) change in body mass (mean ± SE) of woodrats ingesting creosote diets on Day 4 in the feeding trial. Asterisks indicates $p<0.05$ after one-way ANOVA.

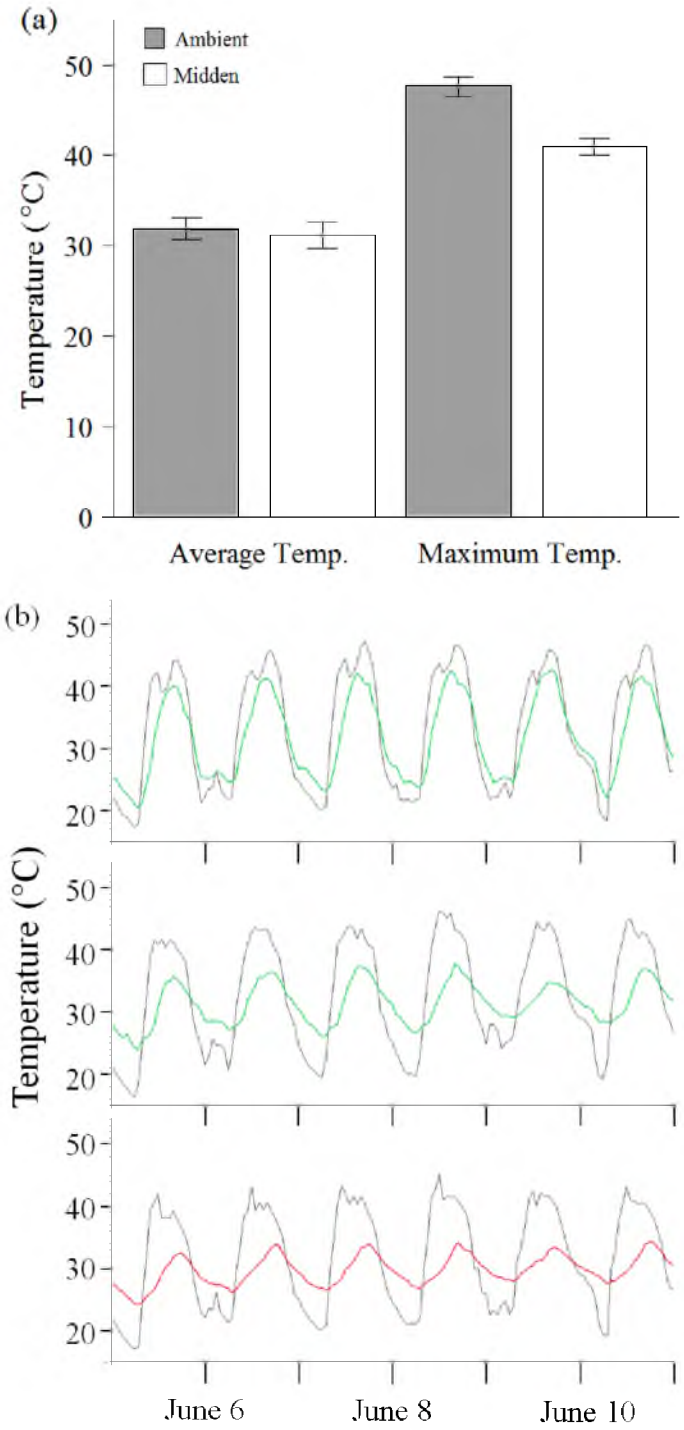

Figure 5.4: (a) Average and maximum monthly temperatures during June – August 2014. Ambient and midden temperatures were collected from three occupied nests. (b) Time-traces of ambient (black line) and midden (colored lines) temperatures from three nests in June 2014. Color indicated plant type at nest (green = creosote bush, red = Joshua tree).

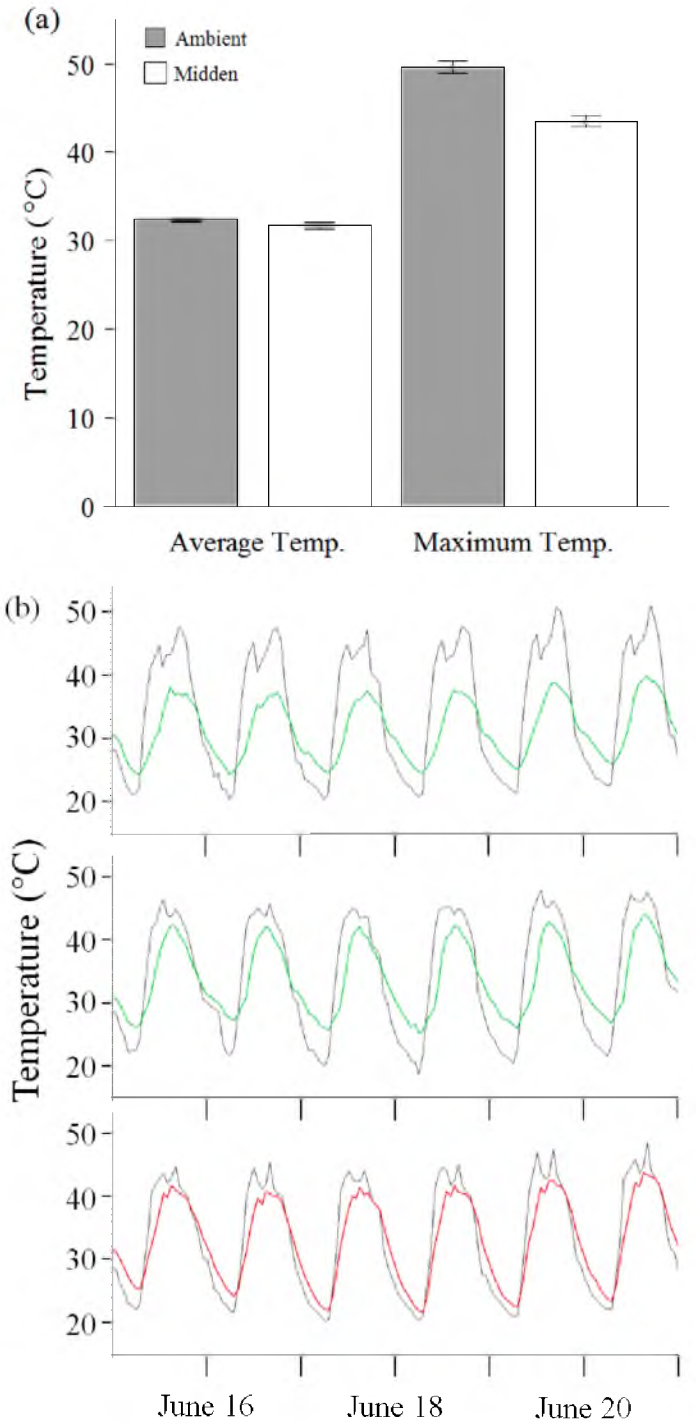

Figure 5.5: (a) Average and maximum monthly temperatures during June – August 2015. Ambient and midden temperatures were collected from three occupied nests. (b) Time-traces of ambient (black line) and midden (colored lines) temperatures from three nests in June 2015. Color indicated plant type (green = creosote bush, red = Joshua tree).

APPENDIX A

THE EFFECT OF TEMPERATURE ON DIET SELECTION IN THE DESERT WOODRAT (*NEOTOMA LEPIDA*)

Introduction

Herbivores are well known to behaviorally regulate ingestion of plant toxins and will reduce their food intake to remain below their physiological limit for detoxification (Torregrossa and Dearing 2009). However, recent evidence for temperature-dependent toxicity posits that herbivores experience a reduced tolerance to plant toxins at warmer temperatures (Kurnath et al. 2016). Such a decrease in the physiological capacity to handle plant toxins may result in a behavioral shift by the herbivore to incorporate fewer plant toxins into its diet at higher ambient temperatures. In fact, such temperature-mediated shifts in diet selection have been documented in the white-throated woodrat and the European starling (Dearing et al. 2008, Chatelain et al. 2013). In the present study, I compared voluntary food intake of desert woodrats at three ambient temperatures by simultaneously presenting two diets differing in creosote resin concentration. I predicted that animals at the higher temperatures would ingest absolutely and relatively less creosote resin than animals at the lower temperatures.

Methods

Animals and plant material were collected from Lytle Ranch Preserve in southern Utah during May 2012 and May 2014 (N=30 woodrats). Animals were housed at the University of Utah in shoebox cages (48x27x20 cm) at room temperature (23-25°C) on a 12L:12D cycle for at least three weeks before experiments. Afterwards, animals were divided into three temperature groups (21°C, 26°C, 31°C) and were acclimated to one temperature for 14 days. Room temperatures were monitored with HOBO data loggers (Onset) throughout the entire study. A cage and diet acclimation period of two days followed, whereby animals were placed in shoebox cages outfitted with two feeder hoods (Lab Products). During this time, woodrats received powdered rabbit chow (Harlan Laboratories) amended with creosote resin at a concentration of 1% (diet preparation as per Kurnath et al. 2016) that was accessible in both feeder hoods. Next, animals entered a six-day feeding trial over which all animals were provided a choice of diets differing in creosote resin concentration: a low 1% resin diet and a high 5% resin diet. Diets were presented in alternating hoods during the trials in order to prevent woodrats from associating one feeder hood with one diet type. Food intake, water intake and body mass were measured each day of the feeding trial. Creosote resin intake was calculated as the product of the amount of ingested food per day times the proportion of creosote resin presented in the diet. Animals were removed from the trial if they lost >10% of their starting body mass. Data was log-transformed for normality as necessary. Daily average intake data were analyzed with one-way ANOVAs and Tukey's post hoc tests.

Results

There was no difference in diet selection across temperature treatments. Regardless of temperature, all animals ingested significantly less diet containing 5% creosote resin (Figure A.1). In addition, there were no significant differences in relative creosote resin intake (Table A.1). The only significantly different intake values occurred in comparisons of total food intake between the 21°C and 31°C temperature groups, whereby animals at 21°C ingested more total food than animals at 31°C ($p=0.044$, Table A.1). This pattern was also apparent when comparing absolute food intake of the 1% creosote resin diet ($p=0.038$, Table A.1). There was no hood-bias for absolute food intake during the trial (Table A.1).

Discussion

These results suggest that ambient temperature does not influence diet selection of creosote resin in the desert woodrat, and thus do not support the original hypothesis. Based upon prior work investigating feeding behaviors and meal sizes in woodrats (Sorensen et al. 2005, Torregrossa et al. 2011), it is probable that all woodrats ingested on average 1.4g of the 5% creosote resin diet each night, because the animals were repeatedly sampling the food available in each hood. In addition, the increased food intake documented at 21°C compared to 31°C aligned with results previously reported at similar temperature treatments for the desert woodrat (Kurnath et al. 2016).

The present findings also provide evidence for two interesting points. First, ambient temperatures within the thermal neutral zone of desert woodrats (26°C and 31°C) did not result in significantly different food intake values. Therefore, these results demonstrated

that the effects of temperature could be a threshold or tipping point response, compared to a graded and increasing response across the thermal neutral zone that was originally suggested (Dearing 2013). Secondly, the plant secondary compounds in creosote resin may not have a thermoregulatory benefit. Other work with the white-throated woodrat found that terpenes in juniper do offer a thermoregulatory benefit and help animals maintain costs of staying warm at lower ambient temperatures (McLister et al. 2004, Dearing et al. 2008). Instead, the plant toxins in creosote resin may be difficult to detoxify at any ambient temperature and thus, when provided the choice, desert woodrats will avoid the higher doses of resin.

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References

Chatelain, M., C. G. Halpin, and C. Rowe. 2013. Ambient temperature influences birds' decisions to eat toxic prey. Animal Behaviour 86:733-740.

Dearing, M. D. 2013. Temperature-dependent toxicity in mammals with implications for herbivores: a review. Journal of Comparative Physiology B 183:43-50.

Dearing, M. D., J. S. Forbey, J. D. McLister, and L. Santos. 2008. Ambient temperature influences diet selection and physiology of an herbivorous mammal, Neotoma albigula. Physiological and Biochemical Zoology 81:891-897.

Kurnath, P., N. D. Merz, and M. D. Dearing. 2016. Ambient temperatures affect tolerance to plant secondary compounds in a mammalian herbivore. Proceedings of the Royal Society B 283.

McLister, J., J. Sorensen, and M. Dearing. 2004. Effects of consumption of juniper (*Juniperus monosperma*) on cost of thermoregulation in the woodrats *Neotoma albigula* and *Neotoma stephensi* at different acclimation temperatures. Physiological and Biochemical Zoology 77:305-312.

Sorensen, J. S., E. Heward, and M. D. Dearing. 2005. Plant secondary metabolites alter the feeding patterns of a mammalian herbivore (*Neotoma lepida*). Oecologia 146:415-422.

Torregrossa, A.-M. and M. D. Dearing. 2009. Nutritional toxicology of mammals: regulated intake of plant secondary compounds. Functional Ecology 23:48-56.

Torregrossa, A. M., A. V. Azzara, and M. D. Dearing. 2011. Differential regulation of plant secondary compounds by herbivorous rodents. Functional Ecology 25:1232-1240.

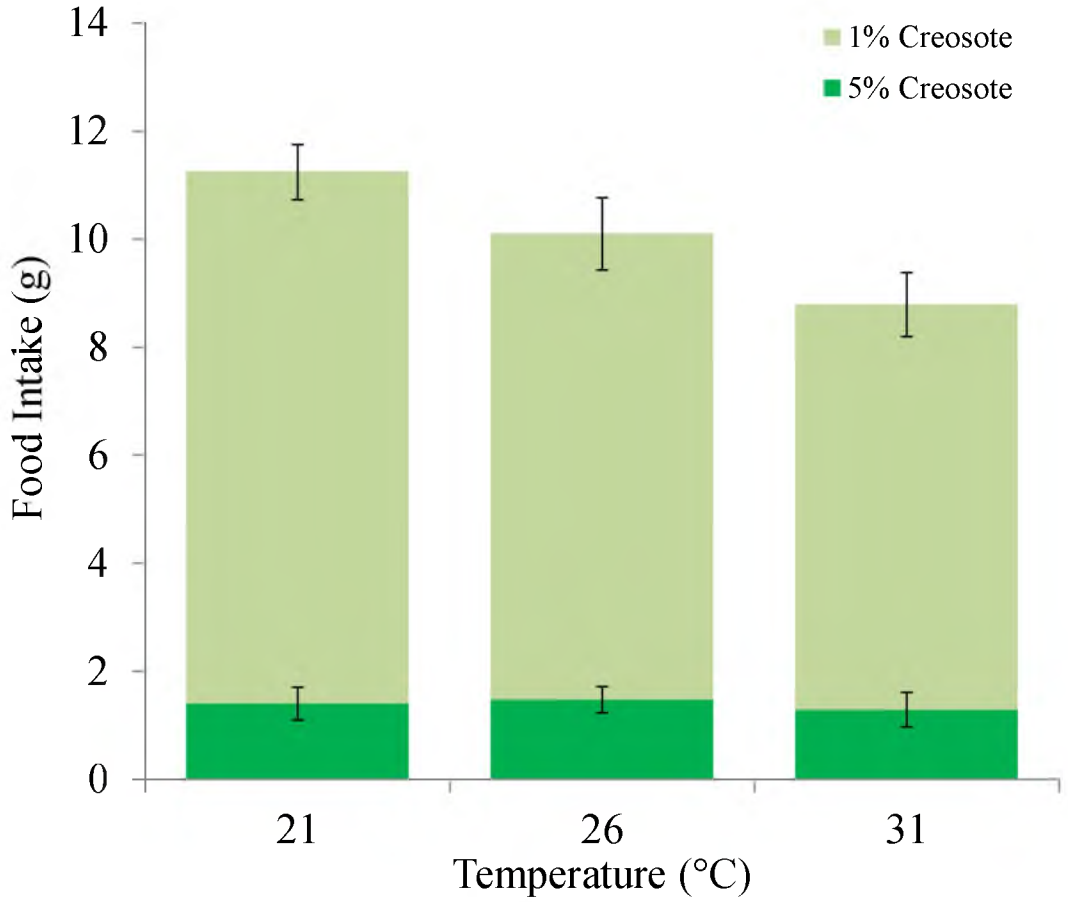

Figure A.1: Choice of food intake (mean ± SE) by woodrats at three temperature treatments. Bar colors represent experimental diets differing in concentration of creosote resin (light green = 1% creosote resin diet and dark green = 5% creosote resin diet).

Table A.1: Summary of ANOVAs comparing intake values and change in body mass in woodrats provided a choice of experimental diets across three temperature treatments. **Bolded values** indicated $p<0.05$ after one-way ANOVA.

	Mean	SE	*F*	*p*	df
Total Food Intake (g)			3.27	**0.055**	2
21°C	11.46	0.49			
26°C	10.3	0.71			
31°C	8.97	0.73			
Total Resin Intake (g)			1.27	0.298	2
21°C	0.1729	0.0148			
26°C	0.1620	0.0139			
31°C	0.1400	0.0176			
Absolute 1% Diet Intake (g)			3.409	**0.048**	2
21°C	9.83	0.51			
26°C	8.62	0.67			
31°C	7.50	0.59			
Absolute 5% Diet Intake (g)			0.121	0.886	2
21°C	1.41	0.30			
26°C	1.48	0.24			
31°C	1.29	0.32			
Relative 1% Diet Intake (%)			0.247	0.783	2
21°C	86.8	0.2			
26°C	84.3	0.1			
31°C	84.6	0.2			
Relative 5% Diet Intake (%)			0.247	0.783	2
21°C	13.2	0.2			
26°C	15.7	0.1			
31°C	15.4	0.2			
Total Water Intake (mL)			0.672	0.519	2
21°C	12.23	1.34			
26°C	12.44	0.78			
31°C	13.9	1.31			
Change in Body Mass (%)			0.776	0.47	2
21°C	-0.33	1.15			
26°C	1.17	0.82			
31°C	-0.58	1.42			
Hood 1 Diet Intake (g)			1.175	0.325	2
21°C	4.44	0.26			
26°C	4.11	0.38			
31°C	3.51	0.68			
Hood 2 Diet Intake (g)			0.878	0.427	2
21°C	3.65	0.31			
26°C	3.59	0.38			
31°C	3.22	0.23			

APPENDIX B

PHYSIOLOGICAL AND BEHAVIOURAL EFFECTS OF FRUIT TOXINS ON SEED-PREDATING VERSUS SEED-DISPERSING CONGENERIC RODENTS

Samuni-Blank, M., I. Izhaki, M.D. Dearing, W.H. Karasov, Y. Gerchman, K.D. Kohl, P. Lymberakis, P. Kurnath, and Z. Arad. 2013. Physiological and behavioural effects of fruit toxins on seed-predating versus seed-dispersing congeneric rodents. Journal of Experimental Biology. Volume 216, pages 3667-3673. Reprinted with permission from the Company of Biologists. The online version is available here: http://jeb.biologists.org/content/216/19/3667.

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RESEARCH ARTICLE

Physiological and behavioural effects of fruit toxins on seed-predating *versus* seed-dispersing congeneric rodents

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SUMMARY

Fleshy, ripe fruits attract seed dispersers but also seed predators. Although many fruit consumers (legitimate seed dispersers as well as seed predators) are clearly exposed to plant secondary compounds (PSCs), their impact on the consumers' physiology and foraging behaviour has been largely overlooked. Here, we document the divergent behavioural and physiological responses to fruit consumption of three congeneric rodent species in the Middle East, representing both seed dispersers and seed predators. The fruit pulp of the desert plant *Ochradenus baccatus* contains high concentrations of glucosinolates (GLSs). These GLSs are hydrolyzed into active toxic compounds upon contact with the myrosinase enzyme released from seeds crushed during fruit consumption. *Acomys russatus* and *A. cahirinus* share a desert habitat. *Acomys russatus* acts as an *O. baccatus* seed predator, and *A. cahirinus* circumvents the activation of the GLSs by orally expelling vital seeds. We found that between the three species examined, *A. russatus* was physiologically most tolerant to whole fruit consumption and even *A. minous*, which is evolutionarily naïve to *O. baccatus*, exhibits greater tolerance to whole fruit consumption than *A. cahirinus*. However, like *A. cahirinus*, *A. minous* may also behaviourally avoid the activation of the GLSs by making a hole in the pulp and consuming only the seeds. Our findings demonstrate that seed predators have a higher physiological tolerance than seed dispersers when consuming fruits containing toxic PSCs. The findings also demonstrate the extreme ecological/evolutionary lability of this plant–animal symbiosis to shift from predation to mutualism and *vice versa*.

Key words: *Acomys*, alanine aminotransferase, bitter taste, body mass, fruits, glucosinolates, liver, pulp, secondary compounds, seeds.

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INTRODUCTION

Fleshy, ripe fruit pulp is a nutritious and easily digestible reward that facilitates seed dispersal by animals (Herrera, 1982; Corlett and Lucas, 1990; Jordano, 2000; Izhaki, 2002a). However, fruits are likely to attract not only seed dispersers but also seed predators because seeds are also a valuable food source (Corlett and Lucas, 1990; Kelt et al., 2004). Ultimately, the nature of the plant–animal interaction is determined by the consumers' fruit eating strategy (Corlett and Lucas, 1990; Jordano, 2000; Dominy and Duncan, 2005).

Variability in fruit eating can be a function of multiple parameters, such as age, satiation/hunger, feeding location, seasonality, or availability of other food or water (Levey, 1987; Corlett and Lucas, 1990; Gautier-Hion et al., 1993; Kaplin and Moermond, 1998; Lambert, 1999; Samuni-Blank et al., 2012). In addition, fruits may contain plant secondary compounds (PSCs) to deter seed predators or alter the behaviour of the consumer (Cipollini and Levey, 1997; Samuni-Blank et al., 2012). In folivorous herbivores, evolutionary pressure has resulted in various behavioural and physiological adaptations for coping with PSCs, thus altering plant–herbivore interactions (McArthur et al., 1991; Dearing et al., 2005; Torregrossa et al., 2011). Similar adaptations to fruit PSCs may also influence fruit eating strategies and thus affect plant–animal interactions.

Glucosinolates (GLSs) are a common class of fruit PSCs. Intact GLSs are generally nontoxic and have a limited effect in defending the plant against herbivores and pathogens. The defensive properties of GLSs are enhanced upon their hydrolysis by the myrosinase enzyme, causing the release of a toxic aglycone (Hopkins et al., 2009). These unstable molecules rearrange into several toxic biological compounds (Kjaer, 1976; Das et al., 2000; Wittstock and Halkier, 2002). Generally, GLSs and myrosinases are compartmentalized to prevent toxicity to the plant, and only mix upon damage to tissue (Rask et al., 2000; Hopkins et al., 2009). The myrosinase–GLS system, also known as the mustard oil bomb, provides plants with an effective defense against generalist herbivores (Wittstock and Halkier, 2002).

The myrosinase–GLS defense system is employed in the fleshy fruit of *Ochradenus baccatus* Delile (Resedaceae), a common inhabitant of wadis and depressions in Israeli desert regions (Bronstein et al., 2007). The fleshy fruits of *O. baccatus* attract a number of consumers because of their high water and sugar content (Bronstein et al., 2007; Spiegel and Nathan, 2007; Spiegel and Nathan, 2011). However, the seeds of *O. baccatus* also represent a valuable nutrient source, as they are rich in protein (~25% of dry mass). To prevent seed predation, the fruits of *O. baccatus* defend

themselves with a unique arrangement of the mustard oil bomb, where GLSs are found in the pulp and the myrosinase enzyme is found in the seeds (Samuni-Blank et al., 2012). Thus, activation of the mustard oil bomb only occurs if consumers crush both seeds and pulp together.

Ochradenus baccatus naturally co-occurs with two congeneric rodent species [*Acomys russatus* (Wagner 1840) and *A. cahirinus* (Geoffrey 1803)] that differ in their interactions with the plant. *Acomys russatus* usually consumes the *O. baccatus* fruit as a whole, acting as a seed predator, while *A. cahirinus* consumes only the pulp and spits the seeds, thus acting as a seed disperser (Samuni-Blank et al., 2012; Samuni-Blank et al., 2013). These interactions result in differential exposure to activated GLSs between species: *A. russatus* consumes activated GLSs by masticating the seeds, while *A. cahirinus* consumes unactivated GLSs by expelling the seeds. We hypothesize that the variation in physiological adaptations to fruit PSCs drive these plant–animal interactions or *vice versa*. We also predict that toxin avoiders will show low physiological tolerance to whole fruit consumption and that the frequency of toxin avoiders within a species will be indicative of its tolerance.

The aim of the present study was to test the effects of fruits PSCs (pulp and seeds) on fruit consumers by preventing behavioural circumvention, while testing the physiological impacts of fruit PSCs on seed dispersers *versus* seed predators. For comparative purposes, we also documented the fruit-eating behaviour and physiological responses to fruit PSCs of the Crete spiny mouse [*A. minous* (Bate 1906)], which is endemic to the Mediterranean island of Crete and evolutionarily naïve to *O. baccatus* fruits. We presented each species with diets containing intact or activated GLSs, and monitored various physiological parameters such as body mass, food intake, dry matter digestibility and serum markers of liver damage. We predicted that the seed predator, *A. russatus*, would be physiologically more adapted to consume activated toxins, as it regularly does so in the wild. We also predicted that consumption of food containing activated GLSs would result in liver damage.

MATERIALS AND METHODS

Fruit collection

Fruits were collected from the Almog junction site (31°48′N, 35°27′E) located near the Dead Sea and kept at −20°C. For experiments in which separation of pulp and seeds was needed, fruits were kept at 4°C and pulp was manually separated from the seeds within 2 days of collection and then kept separately at −20°C. Stored fruits maintained active myrosinase and GLS content (Samuni-Blank et al., 2012). *Ochradenus baccatus* fruiting occurred year-round, with high peaks in May and November 2008–2012. Whole fruits and fruit pulp were thawed before use.

Animals and maintenance

The experimental protocols were approved by the University of Haifa Committee of Animal Experimentation (permit number 096/08). *Acomys cahirinus* [but see Barome et al. and Volobouev et al. (Barome et al., 2001; Volobouev et al., 2007) for a discussion on the *A. cahirinus–dimidiatus* complex) and *A. russatus* were from captive breeding colonies (at least three generations in captivity) originally established from individuals trapped in the vicinity of the Dead Sea (31°28′N, 35°23′E). Animals were maintained at the Department of Biology and Environment at the University of Haifa, Oranim. Adult individuals of *A. minous* were live-trapped in the vicinity of Heraklion, Crete, a few days prior to the experiment using Sherman folding traps placed under rocks. All captured *A. minous* individuals were maintained at the Natural History Museum of Crete (Ministry of Environment, Energy and Climate Change, permit number 117272/586).

Prior to experiments, animals were fed rodent chow (Koffolk serial no. 19510, Tel Aviv, Israel) and fresh carrots as a source of free water, *ad libitum*. During the experiments, animals were housed individually in standard mouse cages (21×31×13 cm) in a temperature-controlled room (25±2°C) under a 12 h:12 h light:dark cycle. All animals were naïve to *O. baccatus* prior to the experiment.

Fruit-eating behaviour of *A. minous*

To determine the fruit-eating behaviour of *A. minous*, we placed each single animal (N=16) in a cage with five fruits overnight. The cage floor was examined the next morning for intact fruit parts (pulp or seeds) and the fruit-eating behaviour was classified as one of three types: (1) 'whole fruit' – eating pulp and seeds simultaneously and leaving no remains; (2) 'pulp' – eating the pulp and leaving the seeds; and (3) 'seed' – eating the seeds and leaving the pulp. Individuals that exhibited more than one fruit-eating behaviour were classified according to their dominant strategy. For example, if one individual ate two whole fruits and only the seeds of the remaining three fruits, then the behaviour was classified as 'seed'. We define avoiders as individuals that consume either pulp or seeds, but not both. We define confronters as individuals that consume the whole fruit, pulp and seeds simultaneously. We recorded the number of intact seeds dropped to the cage floor as well as the number of intact defecated seeds (in the feces). Similar experiments were previously performed on the two other *Acomys* species (Samuni-Blank et al., 2013).

Physiological effects of fruit toxins

To examine the physiological effects of different fruit eating strategies on the three rodent species, we performed controlled feeding trials. Diets included rodent chow (Koffolk serial no. 19510) mixed with homogenized pulp (pulp) or pulp and crushed seeds (mash; Table 1). The fruit pulp was free of myrosinase and contained only intact GLSs. The mash diet combined the GLSs from the pulp and the myrosinase enzyme from the seeds and therefore contained activated GLSs. To allow the animals to acclimate to the fruit diet, they were fed a 25% fruit diet (pulp or mash; wet weight) for 1 day combined with 75% chow, followed by 50% fruit mixed with 50% chow for three additional days. Wet food pellets (~50% water content) were prepared from the mashed food and given to the rodents within an hour of preparation (N=6–8 individual rodents per treatment).

Each experiment lasted 4 days. During the experiment, body mass and food intake were measured daily. Excreta and food leftovers were collected every day from the plastic cage floor, dried (50°C for 24 h) and weighed. Dry matter (DM) digestibility of food consumed by an animal was calculated from its DM food intake and fecal DM output as: DM digestibility=(DM food intake−fecal DM output)/DM food intake.

The effect of activated GLSs on the liver was studied through measurements of liver enzymes and liver function test. On the last

Table 1. Diet components (%)

		Mash	Pulp
Day 1	Chow	50	50
	Water	25	25
	Pulp	23	25
	Seeds	2	0
Days 2–4	Chow	50	50
	Water	0	0
	Pulp	46	50
	Seeds	4	0

Table 2. Summary of the fruit-eating behaviour of the three congeneric species of *Acomys*

	N	Pulp (%)	Seed (%)	Whole (%)	Reference
A. cahirinus	43	76.7	4.6	18.6	Samuni-Blank et al., 2013
A. minous	11*	9.1	54.5	36.4	Present study
A. russatus	43	11.6	0	88.4	Samuni-Blank et al., 2013

*Five other individuals did not consume any part of the fruit and were excluded.

day of the experiment, animals were euthanized with CO_2 and immediately dissected. Blood samples were collected by heart puncture and centrifuged (15 min at 600 ***g***). Blood serum was collected and stored at –20°C. Samples were analyzed for alkaline phosphatase (ALP) and alanine aminotransferase (ALT). ALT is an intracellular enzyme of hepatocytes and its appearance in blood is indicative of possible liver damage. *Acomys cahirinus* and *A. russatus* samples were also analyzed for albumin, gamma-glutamyl transpeptidase (GGT), total bilirubin and direct bilirubin. Serum levels of all these enzymes and proteins are known to increase when hepatobiliary damage occurs (Ozer et al., 2008). All samples were analyzed at the Laboratory of Clinical Biochemistry in Rambam Medical Center, Haifa.

Statistical analyses

To test for differences between the fruit-eating behaviour of *A. minous*, we used a one proportion *Z*-test. Physiological responses on the last day of the diets (body mass, DM digestibility, liver enzymes, etc.) were compared using two-way ANOVA (with species and diet as main effects) followed by Tukey's honestly significant difference (HSD) and linear regression. We also used *t*-tests to compare the slopes of two regression lines. The data were tested for normality prior to statistical comparisons. To evaluate the dispersion of individuals' values from the mean values within each of the species, we calculated the coefficient of variation (CV=100×s.d./mean; %) of the three *Acomys* species under the two diet treatments. Sample size (N=5–6 per species) was kept constant for each of the parameters within each of the diets. For all CV calculations, we used the same sample size; in cases of unequal sample sizes, values from larger groups were removed at random.

In all cases, significance level was set at P<0.05. All data were reported as means ± s.e.m. Statistical analysis was conducted using SPSS 19.0 (IBM, Armonk, NY, USA).

RESULTS

Fruit-eating behaviours

Acomys russatus exhibited the 'whole fruit' behaviour (confronter, consumed pulp and seeds mashed together; movie available at: http://y2u.be/RcLDPst87vs) whereas *A. cahirinus* exhibited the 'pulp' behaviour (avoider, consumed the pulp and spat the seeds; movie available at: http://y2u.be/25XI_mtglPU) (Samuni-Blank et al., 2013). Five individuals of *A. minous* left the fruit untouched, and were not included in the analysis. Interestingly, the most common fruit-eating behaviour of *A. minous* differed from that of the other two *Acomys* species (Table 2). Eight out of 11 individuals employed, at least once, the 'seed' behaviour, by making a hole in the pulp, eating the seeds and leaving the pulp on the cage floor (movie available at: http://y2u.be/yvHL7oA0HbM). The 'seed' behaviour was the dominant behaviour for six of these eight individuals. From these six individuals, four individuals exclusively exhibited the 'seed' (avoider) behaviour and the remaining two individuals alternated between the 'seed' and 'whole fruit' behaviours.

A smaller proportion of *A. minous* (36.6%) preferred the 'whole fruit' (confronter) behaviour (movie available at: http://y2u.be/ghVA7Ibhu8c). Half used the 'seed' strategy exclusively and the others alternated between 'seed' and 'whole fruit' behaviour. Only a single individual used only the 'pulp' (avoider) behaviour (movie available at: http://y2u.be/rb1vC3EbnP8). There was no significant difference between these feeding behaviours (avoider *versus* confronter; Z=0.89, N=11, n.s.). For all individuals, no intact seeds were found in the feces. Overall, we characterized *A. minous* as avoider because more than 50% of the individuals avoided the consumption of the whole fruit and its activated PSCs.

Physiological effects of fruits

Body mass

Final body mass (% of initial) differed significantly among species ($F_{2,39}$=29.5, P<0.001) and between diets ($F_{1,39}$=32.5, P<0.001). In addition, there was a marginally significant diet×species interaction ($F_{2,39}$=3.1, P=0.05). *Post hoc* Tukey's HSD tests showed that on pulp diet, *A. cahirinus* and *A. russatus* maintained the lowest and highest body mass, respectively (P<0.05). Similarly, when fed mash diet, *A. cahirinus* lost significantly more (~20%) of its initial body mass (P<0.05), compared with *A. minous* and *A. russatus*, which lost ~15% and ~10%, respectively (Table 3).

Food intake

DM food intake (%body mass day^{-1}) differed significantly among species ($F_{2,39}$=26.4, P<0.001) and between diets ($F_{1,39}$=23.9,

Table 3. Summary of the feeding trials: body mass (% of initial), dry matter intake (% body mass day^{-1}) and dry matter digestibility (%) of *A. cahirinus*, *A. minous* and *A. russatus* on day 4 of the trial

Parameter	Diet	*A. cahirinus*	*N*	*A. minous*	*N*	*A. russatus*	*N*
Body mass	Pulp	85.0±1.2^{c,d}	8	92.0±2.3^{b}	6	99.5±1.3^{a}	8
	Mash	81.8±0.7^{d}	8	84.8±1.7^{c,d}	7	89.2±1.3^{b,c}	8
Food intake	Pulp	3.3±0.9^{b}	8	5.9±1.0^{a,b}	6	8.6±0.5^{a}	8
	Mash	0.6±0.2^{c}	8	5.3±0.7^{b}	7	4.1±0.6^{b}	8
Digestibility$^{n.s.}$	Pulp	84.9±2.0	8	82.9±2.1	6	81.3±1.1	8
	Mash	80.3±5.6	6	80.6±2.6	7	81.8±2.0	8

Similar letters adjacent to means indicate no significant difference (P>0.05) among the means (two-way ANOVA followed by Tukey's HSD). n.s., not significant. Data are means ± s.e.m.

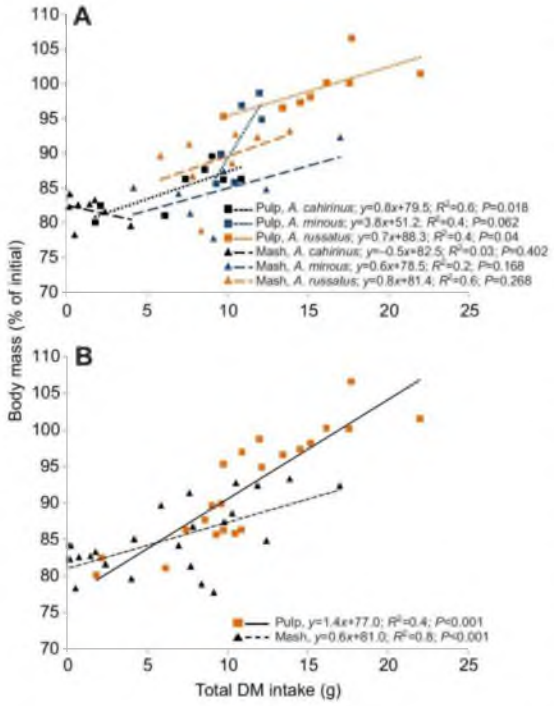

Fig. 1. Change in body mass as a function of total dry matter (DM) intake over all 4 days of the experiment. (A) *Acomys cahirinus* (*N*=16), *A. minous* (*N*=13) and *A. russatus* (*N*=16) feeding on pulp or mash of *Ochradenus baccatus*. (B) Change in body mass as a function of DM intake on the pulp *versus* the mash diet, for all three species combined.

P<0.001). There was a significant interaction between species and diet ($F_{2,39}$=4.1, P<0.05). *Post hoc* Tukey's HSD tests showed that *A. minous* maintained DM intake on the mash diet, while the other two species exhibited a significant decrease (P<0.05) in DM intake on the mash diet compared with the pulp diet. In addition, *A. cahirinus* DM intake on the mash diet was significantly (P<0.05) lower than that of the other two rodents (Table 3).

Digestibility

There were no significant differences in DM digestibility (Table 3) among species ($F_{2,37}$=0.09, n.s.) or between diets ($F_{1,37}$=1.03, n.s.), nor was there a significant diet×species interaction ($F_{2,37}$=0.49, n.s.).

There was a positive correlation between body mass at the end of the trial (% of initial) and total DM food intake for *A. cahirinus* and *A. russatus*, and a marginally positive correlation for *A. minous*, while on the pulp diet (Fig. 1A). There were no significant correlations between intake and final body mass for any species while on the mash diet. The slopes of regression lines differed significantly for pulp and mash diets in all three *Acomys* species combined (t_{43}=3.02, P<0.01; Fig. 1B).

Serum biochemical assay

To our knowledge, we were the first to document serum markers of liver damage of any *Acomys* species. Serum ALT activity differed significantly by diet ($F_{1,38}$=7.9, P<0.01), but not by species ($F_{2,38}$=0.1, n.s.), and there was a significant diet×species interaction ($F_{2,38}$=3.6, P<0.05; Fig. 2A). Inspecting the figure, it was apparent that serum ALT was approximately twice as high in *A. cahirinus* and *A. minous* when eating mash as compared with pulp, whereas *A. russatus* showed no change on the two diets. Despite the significant effects overall in the ANOVA, none of the *post hoc* pairwise comparisons (Tukey's HSD tests) were significant.

There was a significant difference in serum ALP activity (Fig. 2B) among species ($F_{2,36}$=8.9, P=0.001) but not between diets ($F_{1,36}$=1.8, n.s.), nor was there a diet×species interaction ($F_{2,36}$=1.6, n.s.). *Post hoc* Tukey's HSD tests showed that *A. russatus* had significantly lower ALP levels than the other two species (P<0.05). All other comparisons were not significant.

For *A. cahirinus* and *A. russatus*, GGT activity was significantly different among species ($F_{1,28}$=11.7, P=0.002) but not between diet treatments ($F_{1,28}$=0.8, n.s.), and the diet×species interaction was not significant ($F_{1,28}$=0.4, n.s.). For albumin, total bilirubin and direct bilirubin, there were no significant differences (P>0.05) between diet treatments or among species, nor was there a significant diet×species interaction (Table 4).

Coefficient of variation

The CV of all parameters measured for each of the three species and the two diets revealed that for eight out of 10 different CV values, *A. russatus* had the lowest scores (Table 5).

DISCUSSION

The mustard oil bomb products are known to have numerous physiological effects on animals, such as reduction in growth and in food intake, as well as damage to the liver (Duncan and Milne, 1992; Duncan and Milne, 1993; Kim et al., 1997; Sorensen et al., 2001). In the present study, we demonstrated that the activated GLSs of *O. baccatus* differentially affected the performance of seed dispersers and predators.

Two of the three studied species, *A. cahirinus* and *A. russatus*, were previously reported to exhibit very different feeding

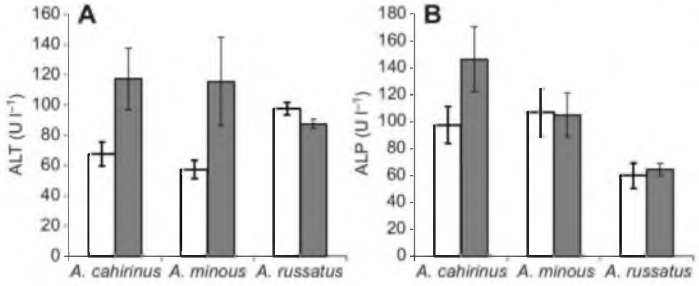

Fig. 2. Serum biochemical assay of *A. cahirinus* (*N*=8 for each diet), *A. minous* (*N*=5–6 for each diet) and *A. russatus* (*N*=8 for each diet) on pulp (white bars) and mash (grey bars) *O. baccatus* fruits for (A) alanine aminotransferase (ALT) and (B) alkaline phosphatase (ALP). Data are presented as means ± s.e.m.

Table 4. Markers of liver damage of *A. cahirinus* (*N*=8 for each diet) *A. russatus* (*N*=8 for each diet) on different diets

Parameter	Diet	*A. cahirinus*	*A. russatus*
GGT ($U l^{-1}$)	Pulp	12.3±1.4	8.9±1.7
	Mash	12.0±0.8	7.0±0.5
Albumin ($g dl^{-1}$)	Pulp	1.1±0.0	1.2±0.1
	Mash	1.1±0.0	1.1±0.0
Total bilirubin ($mg dl^{-1}$)	Pulp	0.1±0.0	0.1±0.0
	Mash	0.1±0.0	<0.05
Direct bilirubin ($mg dl^{-1}$)	Pulp	<0.01	<0.01
	Mash	<0.01	<0.01

GGT, gamma-glutamyl transpeptidase. Data are means ± s.e.m.

Table 5. Coefficient of variation (%) of the three *Acomys* species in the two diet treatments of the different parameters: body mass, dry matter intake, dry matter digestibility, alkaline phosphatase (ALP) and alanine aminotransferase (ALT)

Parameter	Diet	*A. cahirinus*	*A. minous*	*A. russatus*
Body mass	Pulp	3.07	6.15	**1.94**
	Mash	**1.92**	5.92	5.88
Food intake	Pulp	68.93	38.74	**17.41**
	Mash	41.36	**37.18**	45.23
Digestibility	Pulp	7.77	6.29	**2.21**
	Mash	17.04	9.30	**6.38**
ALP	Pulp	29.20	37.38	**11.3**
	Mash	44.82	34.46	**20.57**
ALT	Pulp	40.26	25.95	**13.18**
	Mash	49.50	62.07	**9.87**

The lowest score for each test within each diet is marked in bold.

behaviours: *A. cahirinus* separate the pulp from the seeds to avoid GLS activation while *A. russatus* consume the whole fruit, pulp and seeds. Here, we report that a third species, *A. minous*, evolutionary naïve to *O. baccatus* and closely related to *A. cahirinus*, showed a different dominant behaviour: puncturing a hole in the pulp and consuming only the seeds or consuming the whole fruit. This species tended to avoid the consumption of hydrolyzed GLSs (like *A. cahirinus*), but at the same time acted as a seed predator (like *A. russatus*).

In line with its behaviour, *A. minous* also incurred greater physiological effects than *A. russatus*. The physiological results were consistent with each of the species' fruit-eating strategies: species with a high percentage of PSC avoiders, i.e. individuals spitting seeds or eating only the seeds (Table 2), exhibited a more negative effect of the mash diet on body mass maintenance. After 4 days on the mash diet, *A. russatus* (the confronter) maintained ~90% of its initial body mass, while avoiders *A. minous* and *A. cahirinus* (the seed disperser) maintained only ~85% and ~80% of their initial body mass, respectively. Change in body mass was correlated with food consumption (Fig. 1B) and all species consumed more DM on pulp diet than on mash diet (Table 3).

Although *A. minous* maintained DM intake when feeding on the mash diet, individuals eating that diet lost significantly more body mass than those eating only pulp. This mass loss may stem from the higher energetic costs of detoxifying the hydrolyzed products of the GLSs. Detoxification costs are relatively high, and can be as high as 45% of the basal metabolic costs in specialist herbivores (Sorensen et al., 2005). Accordingly, positive correlations between body mass and food intake for each species were found only on the pulp diet (Fig. 2A). Detoxification costs may also explain the different slopes of the regression lines for the pulp and the mash diets across all *Acomys* species (Fig. 2B).

The very low DM intake of *A. cahirinus*, resulting in significant loss of body mass when feeding on the mash diet, could be explained by the bitter taste of the mustard oil bomb, which serves as a deterrent (Fenwick et al., 1983a; Fenwick et al., 1983b; Garcia-Bailo et al., 2009). Indeed, taste is a valuable tool for estimating food quality, and bitter taste perception prevents animals from ingesting toxic compounds (Chandrashekar et al., 2000; Nelson et al., 2001; Mueller et al., 2005; Chandrashekar et al., 2006). Previous studies have demonstrated that rodents are deterred by bitter taste and also that bitter taste inhibits food intake (Glendinning et al., 1990; Scalera, 1992). However, different mice species may have different taste sensitivities (Glendinning et al., 1990). This could partly explain the higher DM intake values and thus the maintenance of higher body mass of *A. russatus* and *A. minous* when feeding on a mash diet. Also, as individuals of *A. minous* in this study were captured in the wild and thus were exposed to a wider range of diets and environmental changes, it is reasonable that they will show a greater tolerance compared with captive-bred *A. cahirinus*.

Fleshy ripe pulp is used by plants as a nutritious and easily digestible reward for seed-dispersing animals (Herrera, 1982; Corlett and Lucas, 1990; Jordano, 2000; Izhaki, 2002b). We propose that digestibility did not differ between the pulp and mash diets because of the low percentage of seeds (~8%; wet weight) compared with pulp (~92%) in *O. baccatus* fruits.

As an index of the toxicity of the diets, we tested for serum levels of a set of enzymes and proteins known to increase when hepatobiliary damage occurs [ALT, ALP, GGT, albumin and bilirubin (Ozer et al., 2008)]. Toxins can cause acute liver cell necrosis; upon damage to liver cells, aminotransferases enzymes are released into the bloodstream and their activity is elevated in the serum (Ozer et al., 2008; Hyder et al., 2013). Indeed, for *A. cahirinus* and *A. minous*, ALT levels were almost twofold higher on the mash diet than on the pulp diet. For *A. cahirinus* there was also an increase in ALP levels on the mash diet. However, on mash diet, *A. cahirinus* refused to eat (total consumption over 4 days of the experiment was less than 1.5 g) and lost almost 20% of its initial body mass. Severe weight loss is known to be associated with nonalcoholic fatty liver disease (NAFLD) (Salt, 2004). Mice with NAFLD have been shown to possess significantly higher levels of ALT and ALP, but not albumin (Wang et al., 2011). Thus, the severe weight loss on mash diet can also be the reason for the elevated levels in *A. cahirinus* and *A. minous* (who lost more than 15% of its initial body mass), in which the majority of individuals would typically avoid the consumption of the whole fruit and its activated PSCs.

The seed predator *A. russatus* displayed the least variability (i.e. lowest CVs) out of all the study species while on different diets and within the various parameters. In addition, *A. russatus* appeared more tolerant to *O. baccatus* diets in terms of defending body mass when feeding on the mash diet, which contained the toxic components of the mustard oil bomb. This result is consistent with other studies showing the ability of *A. russatus* to maintain body mass under various conditions (Shkolnik and Borut, 1969; Kam and Degen, 1993; Gutman et al., 2006). Physiologically, the unique adaptive mechanisms of the diurnal *A. russatus* for desert survival (Haim and Borut, 1981; Haim et al., 1994; Haim et al., 2005; Ehrhardt et al., 2005; Levy et al., 2011) may also explain their low variability under the different treatments across various examined parameters.

Despite more than three generations of *O. baccatus* naïvety, the seed predator, *A. russatus*, was the least affected by fruit PSCs,

suggesting its higher physiological tolerance to the activated GLSs, while *A. cahirinus*, the seed disperser, was most negatively affected. The ability of the wild-caught, naïve *A. minous* and the captive-bred *A. cahirinus* to behaviourally circumvent the activation of the GLSs, and the physiological ability of *A. russatus* to tolerate the mustard oil bomb, demonstrate the wide ecological/evolutionary lability of this plant–animal symbiosis to shift from predation to mutualism and *vice versa*.

LIST OF ABBREVIATIONS

ALP	alkaline phosphatase
ALT	alanine aminotransferase
DM	dry matter
GGT	gamma-glutamyl transpeptidase
GLS	glucosinolate
NAFLD	nonalcoholic fatty liver disease
PSC	plant secondary compound

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AUTHOR CONTRIBUTIONS

M.S.-B., I.I., M.D.D., W.H.K. and Z.A. conceived the study; M.S.-B., I.I. and M.D.D. designed the study; M.S.-B., M.D.D., K.D.K., W.H.K., P.K. and Z.A. carried out the experiments; M.S.-B., I.I. and W.H.K. analysed the data; M.S.-B., Y.G. and P.L. interpreted the findings. M.S.-B. wrote the paper and all authors contributed substantially to article revision.

COMPETING INTERESTS

No competing interests declared.

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REFERENCES

Barome, P. O., Lymberakis, P., Monnerot, M. and Gautun, J. C. (2001). Cytochrome *b* sequences reveal *Acomys minous* (Rodentia, Muridae) paraphyly and answer the question about the ancestral karyotype of *Acomys dimidiatus*. *Mol. Phylogenet. Evol.* **18**, 37-46.

Bronstein, J. L., Izhaki, I., Nathan, R., Tewksbury, J. J., Spiegel, O., Lotan, A., Altstein, O., Dennis, A. J., Schupp, E. W. and Green, R. J. (2007). Fleshy-fruited plants and frugivores in desert ecosystems. In *Seed Dispersal: Theory and its Application in a Changing World* (ed. A. J. Dennis, E. W. Schupp, R. J. Green and D. W. Westcott), pp. 148-177. Wallingford: CAB International.

Chandrashekar, J., Mueller, K. L., Hoon, M. A., Adler, E., Feng, L., Guo, W., Zuker, C. S. and Ryba, N. J. P. (2000). T2Rs function as bitter taste receptors. *Cell* **100**, 703-711.

Chandrashekar, J., Hoon, M. A., Ryba, N. J. and Zuker, C. S. (2006). The receptors and cells for mammalian taste. *Nature* **444**, 288-294.

Cipollini, M. L. and Levey, D. J. (1997). Secondary metabolites of fleshy vertebrate-dispersed fruits: adaptive hypotheses and implications for seed dispersal. *Am. Nat.* **150**, 346-372.

Corlett, R. T. and Lucas, P. W. (1990). Alternative seed-handling strategies in primates: seed-spitting by long-tailed macaques (*Macaca fasicularis*). *Oecologia* **82**, 166-171.

Das, S., Tyagi, A. K. and Kaur, H. (2000). Cancer modulation by glucosinolates: a review. *Curr. Sci.* **79**, 1665-1671.

Dearing, M. D., Foley, W. J. and McLean, S. (2005). The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. *Annu. Rev. Ecol. Evol. Syst.* **36**, 169-189.

Dominy, N. J. and Duncan, B. W. (2005). Seed-spitting primates and the conservation and dispersion of large-seeded trees. *Int. J. Primatol.* **26**, 631-649.

Duncan, A. J. and Milne, J. A. (1992). Effect of long-term intra-ruminal infusion of the glucosinolate metabolite allyl cyanide on the voluntary food intake and metabolism of lambs. *J. Sci. Food Agric.* **58**, 9-14.

Duncan, A. J. and Milne, J. A. (1993). Effects of oral administration of *brassica* secondary metabolites, allyl cyanide, allyl isothiocyanate and dimethyl disulphide, on the voluntary food intake and metabolism of sheep. *Br. J. Nutr.* **70**, 631-645.

Ehrhardt, N., Heldmaier, G. and Exner, C. (2005). Adaptive mechanisms during food restriction in Acomys russatus: the use of torpor for desert survival. *J. Comp. Physiol. B* **175**, 193-200.

Fenwick, G. R., Heaney, R. K. and Mullin, W. J. (1983a). Bitterness in Brussels sprouts (*Brassica oleracea* L. var. *gemnifera*): the role of glucosinolates and their breakdown products. *J. Sci. Food Agric.* **34**, 73-80.

Fenwick, G. R., Heaney, R. K. and Mullin, W. J. (1983b). Glucosinolates and their breakdown products in food and food plants. *Crit. Rev. Food Sci. Nutr.* **18**, 123-201.

Garcia-Bailo, B., Toguri, C., Eny, K. M. and El-Sohemy, A. (2009). Genetic variation in taste and its influence on food selection. *OMICS* **13**, 69-80.

Gautier-Hion, A., Gautier, J.-P. and Maisels, F. (1993). Seed dispersal versus seed predation: an inter-site comparison of two related African monkeys. *Vegetatio* **108**, 237-244.

Glendinning, J. I., Brower, L. P. and Montgomery, C. A. (1990). Responses of three mouse species to deterrent chemicals in the monarch butterfly. I. Taste and toxicity tests using artificial diets laced with digitoxin or monocrotaline. *Chemoecol.* **1**, 114-123.

Gutman, R., Choshniak, I. and Kronfeld-Schor, N. (2006). Defending body mass during food restriction in *Acomys russatus*: a desert rodent that does not store food. *Am. J. Physiol.* **290**, R881-R891.

Haim, A. and Borut, A. (1981). Heat production and dissipation in golden spiny mice, *Acomys russatus*, from two extreme habitats. *J. Comp. Physiol. B* **142**, 445-450.

Haim, A., Yedidia, I., Haim, D. and Zisapel, N. (1994). Photopenodicity in daily rhythms of body temperature, food and energy intake of the golden spiny mouse (*Acomys russatus*). *Isr. J. Zool.* **40**, 145-150.

Haim, A., Alma, A. and Neuman, A. (2005). Body mass is a thermoregulatory adaptation of diurnal rodents to the desert environment. *J. Therm. Biol.* **31**, 168-171.

Herrera, C. M. (1982). Defense of ripe fruit from pests: its significance in relation to plant–disperser interactions. *Am. Nat.* **120**, 218-241.

Hopkins, R. J., van Dam, N. M. and van Loon, J. J. (2009). Role of glucosinolates in insect–plant relationships and multitrophic interactions. *Annu. Rev. Entomol.* **54**, 57-83.

Hyder, M. A., Hasan, M. and Mohieldein, A. H. (2013). Comparative levels of ALT, AST, ALP and GGT in liver associated diseases. *Eur. J. Exp. Biol.* **3**, 280-284.

Izhaki, I. (2002a). Emodin – a secondary metabolite with multiple ecological functions in higher plants (a review). *New Phytol.* **155**, 205-217.

Izhaki, I. (2002b). The role of fruit traits in determining fruit removal in east Mediterranean ecosystems. In *Seed Dispersal and Frugivory: Ecology, Evolution and Conservation* (ed. D. J. Levey, W. R. Silva and M. Galetti), pp. 161-175. Wallingford: CAB International.

Jordano, P. (2000). Fruit and frugivory. In *Seeds: The Ecology of Regeneration in Plant Communities*, 2nd edn (ed. M. Fenner), pp. 125-166. Wallingford: CAB International.

Kam, M. and Degen, A. A. (1993). Effect of dietary performed water on energy and water budgets of two sympatric desert rodents, *Acomys russatus* and *Acomys cahirinus*. *J. Zool.* **231**, 51-59.

Kaplin, B. A. and Moermond, T. C. (1998). Variation in seed handling by two species of forest monkeys in Rwanda. *Am. J. Primatol.* **45**, 83-101.

Kelt, D. A., Meserve, P. L. and Gutierrez, J. R. (2004). Seed removal by small mammals, birds and ants in semi arid Chile, and comparison with other systems. *J. Biogeogr.* **31**, 931-942.

Kim, D. J., Han, B. S., Ahn, B., Hasegawa, R., Shirai, T., Ito, N. and Tsuda, H. (1997). Enhancement by indole-3-carbinol of liver and thyroid gland neoplastic development in a rat medium-term multiorgan carcinogenesis model. *Carcinogenesis* **18**, 377-381.

Kjaer, A. (1976). Glucosinolates in the Cruciferae. In *The Biology and Chemistry of the Cruciferae* (ed. J. G. Vaughan, A. J. Macleod and B. M. G. Jones), pp. 207-219. London: Academic Press.

Lambert, J. E. (1999). Seed handling in chimpanzees (*Pan troglodytes*) and redtail monkeys (*Cercopithecus ascanius*): implications for understanding hominoid and cercopithecine fruit-processing strategies and seed dispersal. *Am. J. Phys. Anthropol.* **109**, 365-386.

Levey, D. J. (1987). Seed size and fruit-handling techniques of avian frugivores. *Am. Nat.* **129**, 471-485.

Levy, O., Dayan, T. and Kronfeld-Schor, N. (2011). Interspecific competition and torpor in golden spiny mice: two sides of the energy-acquisition coin. *Integr. Comp. Biol.* **51**, 441-448.

McArthur, C., Hagerman, A. E. and Robbins, C. T. (1991). Physiological strategies of mammalian herbivores against plant defenses. In *Plant Defenses Against Mammalian Herbivory* (ed. R. T. Palo and C. T. Robbins), pp. 103-114. Boca Raton, FL: CRC Press.

Mueller, K. L., Hoon, M. A., Erlenbach, I., Chandrashekar, J., Zuker, C. S. and Ryba, N. J. P. (2005). The receptors and coding logic for bitter taste. *Nature* **434**, 225-229.

Nelson, G., Hoon, M. A., Chandrashekar, J., Zhang, Y., Ryba, N. J. P. and Zuker, C. S. (2001). Mammalian sweet taste receptors. *Cell* **106**, 381-390.

Ozer, J., Ratner, M., Shaw, M., Bailey, W. and Schomaker, S. (2008). The current state of serum biomarkers of hepatotoxicity. *Toxicology* **245**, 194-205.

Rask, L., Andreasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B. and Meijer, J. (2000). Myrosinase: gene family evolution and herbivore defense in Brassicaceae. *Plant Mol. Biol.* **42**, 93-114.

Salt, W. B., II (2004). Nonalcoholic fatty liver disease (NAFLD): a comprehensive review. *J. Insur. Med.* **36**, 27-41.

Samuni-Blank, M., Izhaki, I., Dearing, M. D., Gerchman, Y., Trabelcy, B., Lotan, A., Karasov, W. H. and Arad, Z. (2012). Intraspecific directed deterrence by the mustard oil bomb in a desert plant. *Curr. Biol.* **22**, 1218-1220.

Samuni-Blank, M., Arad, Z., Dearing, M. D., Gerchman, Y., Karasov, W. H. and Izhaki, I. (2013). Friend or foe? Disparate plant-animal interactions of two congeneric rodents. *Evol. Ecol.* doi:10.1007/s10682-013-9655-x

Scalera, G. (1992). Taste preferences, body weight gain, food and fluid intake in singly or group-housed rats. *Physiol. Behav.* **52**, 935-943.
Shkolnik, A. and Borut, A. (1969). Temperature and water relations in two species of spiny mice (*Acomys*). *J. Mammal.* **50**, 245-255.
Sørensen, M., Jensen, B. R., Poulsen, H. E., Deng, X., Tygstrup, N., Dalhoff, K. and Loft, S. (2001). Effects of a Brussels sprouts extract on oxidative DNA damage and metabolising enzymes in rat liver. *Food Chem. Toxicol.* **39**, 533-540.
Sorensen, J. S., McLister, J. D. and Dearing, M. D. (2005). Plant secondary metabolites compromise the energy budget of specialist and generalist mammalian herbivores. *Ecology* **86**, 125-139.
Spiegel, O. and Nathan, R. (2007). Incorporating dispersal distance into the disperser effectiveness framework: frugivorous birds provide complementary dispersal to plants in a patchy environment. *Ecol. Lett.* **10**, 718-728.
Spiegel, O. and Nathan, R. (2011). Empirical evaluation of directed dispersal and density-dependent effects across successive recruitment phases. *J. Ecol.* **100**, 392-404.
Torregrossa, A. M., Azzara, A. V. and Dearing, M. D. (2011). Differential regulation of plant secondary compounds by herbivorous rodents. *Funct. Ecol.* **25**, 1232-1240.
Volobouev, V., Auffray, J. C., Debat, V., Denys, C., Gautun, J. C. and Tranier, M. (2007). Species delimitation in the *Acomys cahirinus* – *dimidiatus* complex (Rodentia, Muridae) inferred from chromosomal and morphological analyses. *Biol. J. Linn. Soc. Lond.* **91**, 203-214.
Wang, X., Cao, Y., Fu, Y., Guo, G. and Zhang, X. (2011). Liver fatty acid composition in mice with or without nonalcoholic fatty liver disease. *Lipids Health Dis.* **10**, 234.
Wittstock, U. and Halkier, B. A. (2002). Glucosinolate research in the *Arabidopsis* era. *Trends Plant Sci.* **7**, 263-270.

APPENDIX C

EFFECTS OF FRUIT TOXINS ON INTESTINAL AND MICROBIAL BETA-GLUCOSIDASE ACTIVITIES OF SEED-PREDATING VERSUS SEED-DISPERSING RODENTS (*ACOMYS* SPP.)

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Effects of Fruit Toxins on Intestinal and Microbial β-Glucosidase Activities of Seed-Predating and Seed-Dispersing Rodents (*Acomys* spp.)

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ABSTRACT

Plant secondary compounds (PSCs) have profound influence on the ecological interaction between plants and their consumers. Glycosides, a class of PSC, are inert in their intact form and become toxic on activation by either plant β-glucosidase enzymes or endogenous β-glucosidases produced by the intestine of the plant-predator or its microbiota. Many insect herbivores decrease activities of endogenous β-glucosidases to limit toxin exposure. However, such an adaptation has never been investigated in nonmodel mammals. We studied three species of spiny mice (*Acomys* spp.) that vary in their feeding behavior of the glycoside-rich fruit of *Ochradenus baccatus*. Two species, the common (*Acomys cahirinus*) and Crete (*Acomys minous*) spiny mice, behaviorally avoid activating glycosides, while the golden spiny mouse (*Acomys russatus*) regularly consumes activated glycosides. We fed each species a nontoxic diet of inert glycosides or a toxic diet of activated fruit toxins and investigated the responses of intestinal and microbial β-glucosidase activities. We found that individuals feeding on activated toxins had lower intestinal β-glucosidase activity and that the species that behaviorally avoid activating glycosides also had lower intestinal β-glucosidase activity regardless of treatment. The microbiota represented a larger source of toxin liberation, and the toxin-adapted species (golden spiny mouse) exhibited almost a fivefold increase in microbial β-glucosidase when fed activated toxins, while other species showed slight decreases. These results are contrary to those in insects, where glycoside-adapted species have lower β-glucosidase activity. The glycoside-adapted golden spiny mouse may have evolved tolerance mechanisms such as enhanced detoxification rather than avoidance mechanisms.

Keywords: Acomys, digestion, glycosides, gut microbes, plant-animal interactions, plant secondary compounds.

Introduction

Plants produce various types of secondary compounds (PSCs) to deter consumption by animals (Dearing et al. 2005). Glycosides, a class of PSC, are stored in inactive forms by attaching a glucose molecule to a toxic moiety (the aglycone) through a β-glycosidic linkage (Morant et al. 2008). Glycosides in their intact forms are typically physiologically inert but can often be activated by β-glucosidase enzymes produced by plants. These enzymes are usually compartmentalized to avoid release of toxic aglycones within the plant and instead interact with glycosides upon physical disturbance, such as mastication by animals (Morant et al. 2008). The intestinal tracts of animals also contain β-glucosidase enzymes, which are produced by the gut tissue and symbiotic microbes. These β-glucosidase enzymes benefit animals by digesting nutritional compounds such as cellobiose and oligosaccharides (Yapi et al. 2009; Sharf et al. 2010). Additionally, the enzyme lactase phlorizin hydrolase is an intestinal β-glucosidase enzyme that hydrolyzes the milk sugar lactose (Karasov and Douglas 2013). However, these endogenous β-glucosidase enzymes, including lactase phlorizin hydrolase, can also hydrolyze plant glycosides, allowing the aglycones to be absorbed through intestinal tissue (Day et al. 2000; Németh et al. 2003). Thus, the hydrolysis of glycosides by endogenous enzymes represents a mechanism by which animals might inadvertently increase the toxicity of their diets.

In order to avoid the release of toxic compounds, many insects reduce activities of midgut β-glucosidases in response to diets containing glycosides (Lindroth 1988; Desroches et al. 1997; Ferreira et al. 1997; Pankoke et al. 2010, 2012). It is also thought that glycosides are nontoxic to birds due to their low activities of endogenous β-glucosidases (Struempf et al. 1999). However, such a physiological adaptation has never been

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investigated in mammals that naturally consume glycosides. Additionally, the gut microbiota play a large role in the ecology and evolution of their hosts (McFall-Ngai et al. 2013) and have a greater capacity to hydrolyze glycosides than host intestinal tissue (Nakano and Gregory 1995). However, the response of gut microbial β-glucosidase activities to dietary glycosides has not been studied in natural systems.

We investigated the response of endogenous β-glucosidase activities to dietary glycosides in three species of spiny mice (*Acomys* spp.) that differ in their consumption of the glycoside-rich fruit of sweet mignonette (*Ochradenus baccatus* Delile). The principal defense compounds in *O. baccatus* are glucosinolates (Lotan and Izhaki 2013), which contain a sulfur atom between the glucose and aglycone group and must be activated by myrosinase or thioglucoside glucohydrolase enzymes (Kliebenstein et al. 2005). The vertebrate gut apparently has little to nil capacity to hydrolyze glucosinolates (Lessner et al. 2015), and so there is little to no opportunity for vertebrates to limit exposure to these toxic compounds via changes in enzyme activities. However, in addition to glucosinolates, *O. baccatus* produces a number of typical O-glycosides (Barakat et al. 1991; Hussain et al. 2014), which can be acted on by β-glucosidase enzymes (Ketudat Cairns and Esen 2010). Physiological adaptations in the activities of β-glucosidase enzymes have not yet been investigated in vertebrates, though this idea has been suggested (Majak 1991).

Previous work on this system has demonstrated that three species of spiny mice (*Acomys* spp.) vary in their behavioral and physiological adaptations to the toxins of *O. baccatus*. The toxic components of *O. baccatus* fruit are compartmentalized, such that the glycosides are stored in the fruit pulp and glucosidase enzymes are stored in the seeds (Samuni-Blank et al. 2012). Thus, toxic aglycones are released only if a seed predator crushes the seeds while consuming the pulp. In behavioral trials we previously reported that two species of *Acomys*, the common spiny mouse (*Acomys cahirinus* Geoffrey) and Crete spiny mouse (*Acomys minous* Bate), are not likely to activate glycosides, as they prefer to consume only the pulp or seed, respectively, and discard the other component (Samuni-Blank et al. 2013*a*, 2013*b*). The golden spiny mouse (*Acomys russatus* Wagner) crushes the seed and pulp of *O. baccatus* together and is therefore exposed to activated glycosides (Samuni-Blank et al. 2013*a*). Thus, these species interact with *O. baccatus* very differently, with the common spiny mouse acting as seed disperser and the Crete and the golden spiny mice acting as seed predator. Further, when these species are fed diets of mashed fruit and seeds (thus activating the toxins), the common and Crete spiny mice drastically reduce food intake and lose 15%–20% of their body mass, while the golden spiny mouse exhibits higher food intake and only loses ~10% of body mass (Samuni-Blank et al. 2013*b*, 2014). These results suggest that the golden spiny mouse has physiological adaptations for coping with these toxins.

We fed spiny mice nontoxic diets containing only the pulp of *O. baccatus* (pulp diet) or toxic diets containing pulp and crushed seeds (mash diet), which results in activation of glycosides. We measured the intestinal and microbial activities of two α-glucosidases (maltase, sucrase) and two β-glucosidases (salicinase, amygdalinase). We predicted that the toxin-adapted species, the golden spiny mouse, would show lower constitutive intestinal and microbial β-glucosidase activities compared to other spiny mice species. We also predicted that intestinal and microbial β-glucosidase activities would decrease in all species in response to the toxic diet. These predictions are consistent with what has been observed in insects (Lindroth 1988; Desroches et al. 1997; Ferreira et al. 1997; Pankoke et al. 2010, 2012). We also measured α-glucosidase activities (maltase, sucrase) with the expectation that these activities would not be affected by diet, given that they do not interact with glycosides. However, some glycosides have the potential to inhibit digestive enzymes (Silva et al. 2006), and, thus, measurements of α-glucosidases were useful to investigate the possibility of universal changes in enzyme activities, even in nontarget enzymes.

Methods

Animals

All samples were collected from animals used in other studies (Samuni-Blank et al. 2013*b*). While these animals had been in captivity for a period of time, our findings are likely still ecologically relevant. First, the differential feeding behavior of species was observed in both wild and captive-bred individuals (Samuni-Blank et al. 2012, 2013*a*, 2013*b*), suggesting that the differential tolerance to fruit toxins does not change with captivity. Second, interspecific differences in microbial communities are often maintained when animals are brought into captivity (Fraune and Bosch 2007; Kohl et al. 2014*b*). Briefly, individuals of all three species were housed individually in standard mouse cages (21 cm × 31 cm × 13 cm) in a temperature-controlled room (25° ± 2°C) under a 12L:12D cycle. Animals were fed carrots and rodent chow (Koffolk 19510, Tel Aviv, Israel). During the diet trial, animals were fed either a pulp diet (rodent chow mixed with homogenized *Ochradenus baccatus* pulp, which contains only inert, intact glycosides) or a toxic mash diet (rodent chow mixed with pulp and crushed seeds; myrosinase in the seeds activates the pulp toxins). Animals were given a diet of 25% fruit (pulp or mash) for 1 d, followed by 50% fruit (pulp or mash) for 3 d. Sample sizes for the experiment were as follows: common spiny mouse: 8 pulp, 8 mash; Crete spiny mouse: 6 pulp, 7 mash; golden spiny mouse: 8 pulp, 8 mash. Following the trial, animals were euthanized with CO_2 and immediately dissected. Entire contents were extruded from the small intestine, placed on dry ice to freeze, and later stored at −80°C. Next, the small intestine was cut in half longitudinally. Half of the small intestine was placed on dry ice to cause flash freezing and was later frozen at −80°C for storage, while the other half was placed in RNAlater for other experiments. Tissues were transported on dry ice to the University of Utah (Salt Lake City) for enzyme analysis. All protocols were approved by the Committee of Animal Experimentation of the University of Haifa (permit 096/08) and the University of Utah Institutional Animal Care and Use Committee (protocol 10-01013).

Enzyme Assays

We assayed glucosidase activities using a modification of a previously developed colorimetric method (Dahlqvist 1984). Enzyme activities were measured either in intestinal tissue or from gut contents, which estimates microbial β-glucosidase activity (Banks et al. 1994; Nakano and Gregory 1995; Hylla et al. 1998). We measured the activities of two α-glucosidase enzymes that hydrolyze nutrients, maltase and sucrase, to investigate the possibility of universal effects of glycosides on digestive enzymes. Additionally, we measured the hydrolase activities against two β-glycosides, amygdalin and salicin. It should be noted that amygdalinase and salicinase activities are not the direct measurements of activities of specific enzymes but rather the capacity for a number of glucohydrolase enzymes to hydrolyze these substrates. These substrates have been widely used to estimate the β-glucosidase activity toward plant-derived glycosides (Adewusi and Oke 1985; Lindroth 1988; Ferreira et al. 1997; Lessner et al. 2015). Briefly, intestinal tissue or luminal contents were thawed and homogenized in 350 mM mannitol in 1 mM N-2-hydroxyethylpiperazine-N′-2-ethanosulfonic acid (Hepes)-KOH, pH 7.0. Homogenates (30 μL) diluted with 350 mM mannitol in 1 mM Hepes-KOH were incubated with 30 μL of 56 mM maltose, sucrose, amygdalin, or salicin in 0.1 M maleate and NaOH buffer, pH 6.5, at 37°C for 20 min. Next, 400 μL of a stop-develop reagent (GAGO-20 glucose assay kit; Sigma-Aldrich, St. Louis, MO) was added to each tube, vortexed, and incubated at 37°C for 30 min. Last, 400 μL of 12 N H_2SO_4 was added to each tube to stop the reaction. Blank tubes were used to control for endogenous glucose present in tissues. These blank tubes contained the same reagents and tissues as those used for measuring activity, but the substrates were added after the addition of H_2SO_4 to prevent the enzymatic reactions from occurring. Several 200-μL aliquots of the final reactions were transferred to a 96-well plate, and the absorbance was read at 540 nm using a BioTek (Broadview, IL) PowerWave HT microplate spectrophotomer. Protein content was measured using a Bradford assay with a standard curve generated using bovine serum albumin.

Statistics

Activities of β-glucosidase were transformed using a $(x + 1)^{0.5}$ transformation (McDonald 2014). Enzyme activities were compared with two-way ANOVAs, using diet and species as main effects, with JMP 12.0 and an α value of 0.05. Differences among groups were investigated using Tukey's HSD test. Using these same animals, we have previously demonstrated that these species respond differently to the toxic and nontoxic diets in terms of food intake and changes in body mass (Samuni-Blank et al. 2013*b*). Given that food intake and fasting can influence digestive enzyme activities (McNeill and Hamilton 1971), we included the following variables as covariates: total food consumption over the 4-d trial normalized to body mass$^{3/4}$ and percent change in body mass. Also, to investigate whether differences in β-glucosidase activities were specific or perhaps due to universal differences in activities of all glucosidases, we conducted ANCOVAs for salicinase and amygdalase using maltase activity as a covariate. Nonsignificant covariates ($P > 0.05$) were removed from the final analyses.

Results

Measurements of β-glucosidase enzyme activities did not covary with normalized food intake, percent change in body mass, or maltase activities, and thus all covariates were removed from the final models. Intestinal activities of all glucosidases differed significantly between species, with the golden spiny mouse exhibiting the highest activities (table 1; fig. 1). Intestinal activities of α-glucosidases (maltase, sucrase) and amygdalase showed no significant diet effect. There was a significant effect of diet on intestinal salicinase activity (table 1; fig. 1), such that individuals fed the activated mash diet exhibited salicinase activity that was 34%–69% lower than those fed the pulp diet.

There were no significant differences between species or diet treatments on the microbial activities of α-glucosidases (maltase, sucrase) or the β-glucosidase amygdalase (table 1; fig. 2). Interestingly, microbial salicinase activity showed a highly significant diet × species interaction. When feeding on the mash diet, the microbial salicinase activity of the common and Crete spiny mice was 31%–66% lower compared to that of those fed the nontoxic pulp diet, whereas the golden spiny mice fed the toxic mash diet exhibited a 4.8-fold higher microbial salicinase activity compared to animals fed the pulp diet.

Discussion

The physiological responses of β-glucosidase enzymes to dietary glycosides have been studied in a number of insect systems. This study represents the first investigation into these responses in nonmodel species of mammals. We predicted that the toxin-adapted species, the golden spiny mouse (Samuni-Blank et al. 2013*a*), would show lower constitutive intestinal and microbial β-glucosidase activities and that all species would decrease intestinal and microbial β-glucosidase activities in response to dietary glycosides, that is, the activated mash diet. Many of our predictions were not supported. In contrast to our prediction for across-species differences, the golden spiny mouse had the highest activities of intestinal and microbial β-glucosidases. Although intestinal salicinase activity declined as predicted when ingesting the toxic diet, there was no significant effect of diet on amygdalinase activity. Furthermore, microbial β-glucosidase activity declined in the common and Crete spiny mice consuming the mash diet; however, it increased in the golden spiny mouse. Thus, our hypothesis that the golden spiny mouse may limit toxin exposure by lowering rates of intestinal hydrolysis was not supported. Rather, this species may exhibit tolerance mechanisms, such as enhanced hepatic detoxification. We discuss these findings below, as well as potential ecological implications.

Intestinal β-Glucosidases

The golden spiny mouse showed the highest intestinal β-glucosidase activities (salicinase, amygdalinase). This species

Table 1: ANOVA results for intestinal and microbial enzyme activities

	Intestine			Microbiota		
	F	df	*P*	*F*	df	*P*
Maltase:						
Diet	1.62	1, 39	.21	2.06	1, 39	.16
Species	12.33	2, 39	**<.0001**	1.73	2, 39	.19
Diet × species	1.36	2, 39	.27	.07	2, 39	.92
Sucrase:						
Diet	.17	1, 39	.68	.28	1, 39	.60
Species	17.89	2, 39	**<.0001**	3.01	2, 39	.06
Diet × species	.46	2, 39	.63	.02	2, 39	.98
Salicinase:						
Diet	4.26	1, 39	**.046**	1.52	1, 39	.23
Species	3.51	2, 39	**.039**	4.57	2, 39	**.016**
Diet × species	.70	2, 39	.50	6.45	2, 39	**.004**
Amygdalase:						
Diet	2.51	1, 39	.12	.03	1, 39	.87
Species	4.87	2, 39	**.013**	2.12	2, 39	.13
Diet × species	.09	2, 39	.91	.52	2, 39	.60

Note. Salicinase and amygdalinase activities were transformed using a $(x + 1)^{0.5}$ transformation before statistical analysis. Bolded items are statistically significant ($P < 0.05$).

also showed the highest activities of intestinal α-glucosidases (maltase, sucrase). This pattern could suggest that all intestinal glucosidases are under universal regulation in these species. However, β-glucosidase activities did not covary with maltase activity. In addition, the mash diet decreased intestinal salicinase activities but had no effect on maltase or sucrase activities. Thus, it seems more likely that activities of intestinal β-glucosidases are regulated separately from α-glucosidases.

The golden spiny mouse exhibited higher intestinal β-glucosidase activity than the other spiny mouse species, which should serve to increase the bioavailability of toxic aglycones. This result did not support our predictions and is contrary to what has been reported for beetle and lepidopteran larvae, where adaption to dietary glycosides is associated with reduced constitutive β-glucosidase activities (Desroches et al. 1997; Lindroth 1988). However, due to the seed-crushing behavior of the golden spiny mouse, its intestinal tissue and enzymes are more likely to encounter toxic aglycones rather than intact glycosides. Thus, the avoidance mechanism of low intestinal β-glucosidase activity may be at best irrelevant in the golden spiny mouse and at worst disadvantageous because β-glucosidases digest some nutritional compounds such as lactose, cellobiose, and oligosaccharides (Yapi et al. 2009; Scharf et al. 2010). The effects that these changes in enzyme activities might have on the digestion of nutrients require further investigation. The golden spiny mouse may have instead evolved tolerance mechanisms, such as enhanced hepatic detoxification, to cope with high levels of dietary glycosides. The avoidance mechanism is instead observed in the common spiny mouse, which exhibits the lowest activities of intestinal β-glucosidases and also behaviorally avoids toxins by separating the seed and pulp. In this case, low activities of β-glucosidases may reduce its exposure to toxic aglycones.

In all species, we observed a significant decrease in intestinal salicinase activity on the activated mash diet. Again, this response was independent of regulation of maltase and sucrase and so is likely to be a targeted response to dietary glycosides. Lowering intestinal β-glucosidase activities might prevent further exposure to toxins. For this mechanism to be effective, β-glucosidase activities would need to be downregulated in response to glycosides or aglycone molecules. While downregulation of β-glucosidase activities in response to aglycones has not been studied in terms of toxic glycosides, there is precedence for it in other systems. Both dietary pyridoxine-glucoside and the aglycone pyridoxine cause decreases in intestinal β-glucosidase activity in laboratory rats (Nakano and Gregory 1995) and guinea pigs (Banks et al. 1994). Interestingly, the aglycone pyridoxine is more potent at reducing intestinal β-glucosidase activities than pyridoxine-glucoside (Nakano and Gregory 1995), perhaps due to higher rates of absorption (Németh et al. 2003). Concentrations of PSCs in the blood are thought to be important for determining animals' physiological and behavioral responses (McLean and Duncan 2006; Torregrossa and Dearing 2009). Thus, the higher concentrations of easily absorbable, toxic aglycones in the activated mash may underlie the lower intestinal β-glucosidase activities in spiny mice fed this diet. It would be interesting to investigate the mechanisms of regulation of β-glucosidase activity in response to purified glycosides and aglycones in an ecologically relevant context.

Microbial β-Glucosidases

The activity of intestinal microbial β-glucosidase activity per unit protein was 3.5–6 times that of intestinal tissue. These results are similar to findings in rats, where intestinal microbial

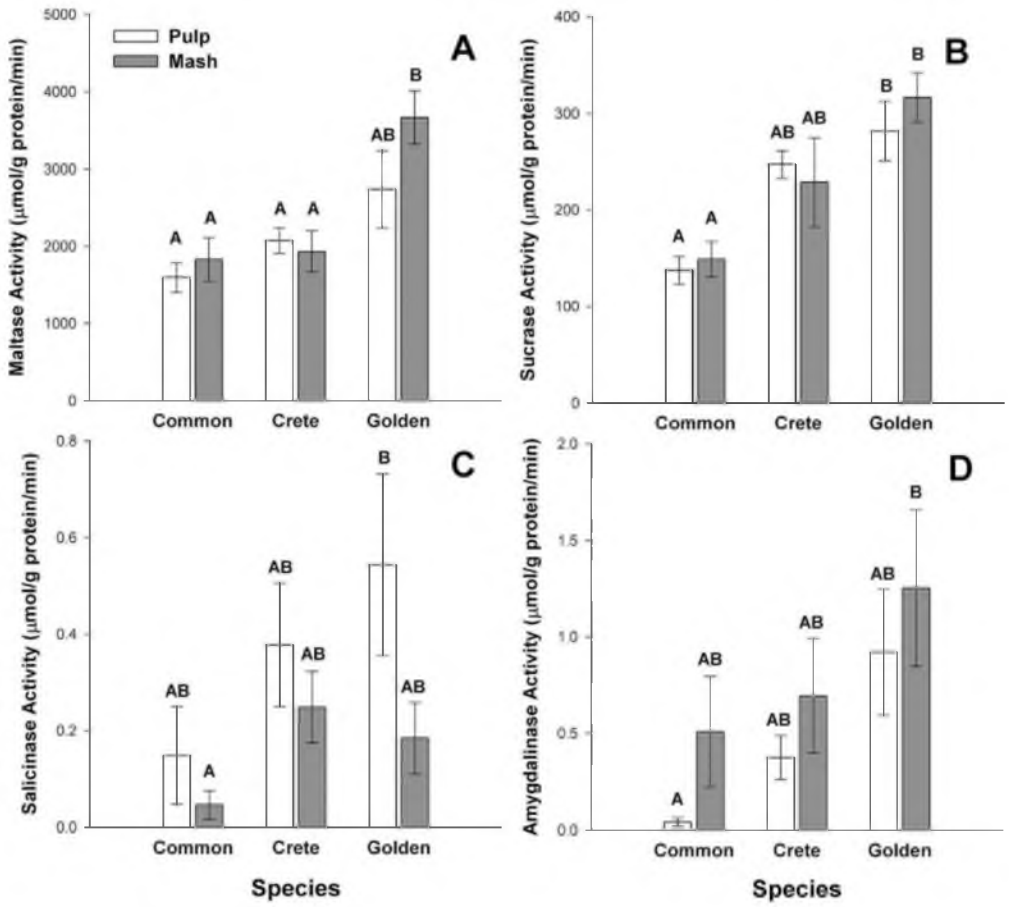

Figure 1. Mean activities of maltase (*A*), sucrase (*B*), salicinase (*C*), and amygdalinase (*D*) from host intestinal tissues of common (*Acomys cahirinus*), Crete (*Acomys minous*), and golden (*Acomys russatus*) spiny mice. Mice were fed either a nontoxic pulp diet or a toxic mash diet. Bars represent means ± SEM. Graphs depict original data, though statistical analyses for salicinase and amygdalinase were conducted on transformed data. Bars not sharing letters are significantly different as determined by Tukey's HSD test.

activity was ~10 times higher than intestinal tissue (Nakano and Gregory 1995). These results are remarkable given that the small intestine harbors the lowest density of microbial cells across the gut (Kohl et al. 2014*a*). The techniques used here have been widely used to estimate both intestinal and microbial β-glucosidase activities (Hylla et al. 1998; Banks et al. 1994; Nakano and Gregory 1995). It could be argued that activity detected in the gut lumen may be driven by the sloughing off of intestinal cells with active enzymes. However, we are confident that we primarily measured microbial activity. First, previous studies have utilized fluorescent staining of the gut lumen of rodents to reveal that it is overwhelmingly dominated by bacteria and not intestinal cells (Johansson et al. 2008). Second, β-glucosidase activities from the intestine and the lumen showed different responses to glycosides, such that intestinal salicinase activity decreased on the toxic diet while luminal activity increased. If luminal activity were primarily driven by intestinal activity, we would predict the same direction of change in these two measurements. However, the possibility exists that luminal activities were partially driven by intestinal enzymes or sloughed mucosal cells. Studies with germ-free mice (those lacking a microbiota) would elucidate the proportion of luminal activity generated by microbes.

In our experiment, we observed a strong diet × species interaction in microbial salicinase activity, such that the common and Crete spiny mice exhibited lower activity when feeding on the mash diet, while the golden spiny mouse exhibited higher activity. Results from the golden spiny mouse follow previous research showing induction of microbial β-glucosidase by both pyridoxine-glucoside and the aglycone pyridoxine (Banks et al. 1994; Nakano and Gregory 1995). Induction of microbial β-glucosidase in a toxin-adapted species may facilitate the microbial metabolism of aglycone molecules (Keppler and Humpf 2005). However, in the common and Crete spiny mice we see lower

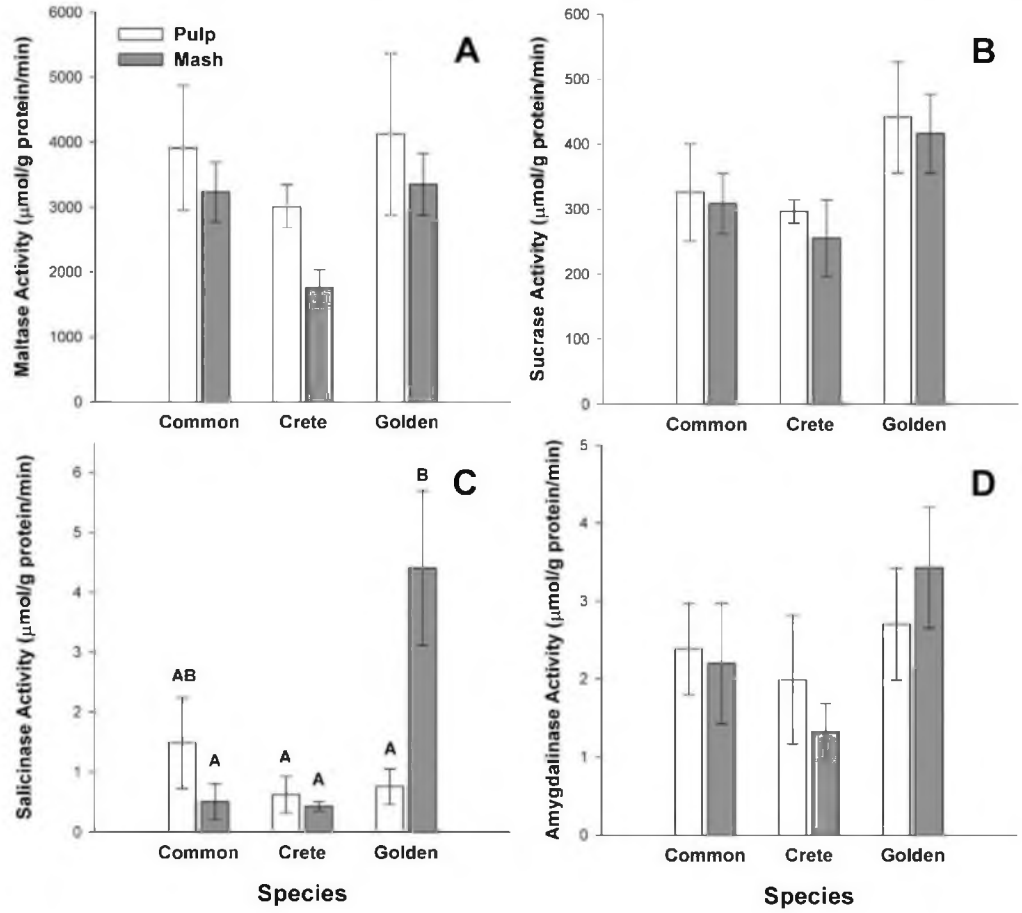

Figure 2. Mean activities of microbial maltase (*A*), sucrase (*B*), salicinase (*C*), and amygdalinase (*D*) in common (*Acomys cahirinus*), Crete (*Acomys minous*), and golden (*Acomys russatus*) spiny mice. Mice were fed either a nontoxic pulp diet or a toxic mash diet. Bars represent means ± SEM. Graphs depict original data, though statistical analyses for salicinase and amygdalinase were conducted on transformed data. Bars not sharing letters are significantly different as determined by Tukey's HSD test.

microbial β-glucosidase activity when feeding on the mash diet. This reduction in activity may benefit the host by limiting hydrolysis of glycosides, allowing them to be excreted in the feces. Hosts exert strong selection on which bacteria flourish in the gut (Rawls et al. 2006), and host experience with plant toxins exerts a selective force on gut microbial community structure (Kohl and Dearing 2012). Thus, each species may house microbial populations that facilitate either tolerance or avoidance of glycosides, a possibility that warrants further study.

Conclusions

Species that behaviorally avoid the liberation of glycosides (by consuming only seeds or only pulp) also avoid endogenous hydrolysis by intestinal and microbial enzymes. The golden spiny mouse readily consumes activated glycosides and shows less physiological avoidance to hydrolysis. Thus, it is likely that the golden spiny mouse has adapted tolerance mechanisms to deal with these toxic compounds. Such adaptations may influence the feeding behavior of the studied species, thus altering their ecological role as either seed dispersers or seed predators.

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Literature Cited

Adewusi S.R.A. and O.L. Oke. 1985. On the metabolism of amygdalin. 2. The distribution of β-glucosidase activity and orally administered amygdalin in rats. Can J Physiol Pharmacol 63:1084–1087.

Banks M.A., D. Porter, W.G. Martin, and J.F. Gregory III. 1994. Dietary vitamin B6 effects on the distribution of intestinal mucosal and microbial β-glucosidase activities toward pyridoxine-5′-β-glucoside in the guinea pig. J Nutr Biochem 5:238–242.

Barakat H.H., A.M.D. El-Mousallamy, A.M.A. Souleman, and S. Awadalla. 1991. Flavonoids of *Ochradenus baccatus*. Phytochemistry 30:3777–3779.

Dahlqvist A. 1984. Assay of intestinal disaccharidases. Scand J Clin Lab Invest 44:173–176.

Day A.J., F.J. Cañada, J.C. Díaz, P.A. Kroon, R. Mclauchlan, C.B. Faulds, G.W. Plumb, M.R.A. Morgan, and G. Williamson. 2000. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. FEBS Lett 468:166–170.

Dearing M.D., W.J. Foley, and S. McLean. 2005. The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. Annu Rev Ecol Evol Syst 36:169–185.

Desroches P., N. Mandon, J.C. Baehr, and J. Huignard. 1997. Mediation of host-plant use by a glucoside in *Callosobruchus maculatus* F. (Coleoptera: Bruchidae). J Insect Physiol 43: 439–446.

Ferreira C., J.R.P. Parra, and W.R. Terra. 1997. The effect of dietary plant glycosides on larval midgut β-glucosidases from *Spodoptera frugiperda* and *Diatraea saccharalis*. Insect Biochem Mol Biol 27:55–59.

Fraune S. and T.C.G. Bosch. 2007. Long-term maintenance of species-specific bacterial microbiota in the basal metazoan *Hydra*. Proc Natl Acad Sci USA 104:13146–13151.

Hussain J., N.U. Rehman, A.L. Khan, L. Ali, J.S. Kim, A. Zakarova, A. Al-Harrasi, and Z.K. Shinwari. 2014. Phytochemical and biological assessment of medicinally important plant *Ochradenus arabicus*. Pak J Bot 46:2027–2034.

Hylla S., A. Gostner, G. Dusel, H. Anger, H.-P. Bartram, S.U. Christl, H. Kasper, and W. Scheppach. 1998. Effects of resistant starch on the colon in healthy volunteers: possible implications for cancer prevention. Am J Clin Nutr 67:136–142.

Johansson M.E., M. Phillipson, J. Petersson, A. Velcich, L. Holm, and G.C. Hansson. 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci USA 105:15064–15069.

Karasov W.H. and A.E. Douglas. 2013. Comparative digestive physiology. Compr Physiol 3:741–783.

Keppler K. and H.-U. Humpf. 2005. Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora. Bioorg Med Chem 13:5195–5205.

Ketudat Cairns J.R. and A. Esen. 2010. β-glucosidases. Cell Mol Life Sci 67:3389–3405.

Kliebenstein D.J., J. Kroymann, and T. Mitchell-Olds. 2005. The glucosinolate-myrosinase system in an ecological and evolutionary context. Curr Opin Plant Biol 8:264–271.

Kohl K.D. and M.D. Dearing. 2012. Experience matters: prior exposure to plant toxins enhances diversity of gut microbes in herbivores. Ecol Lett 15:1008–1015.

Kohl K.D., A.W. Miller, J.E. Marvin, R.I. Mackie, and M.D. Dearing. 2014*a*. Herbivorous rodents (*Neotoma* spp.) harbour abundant and active foregut microbiota. Environ Microbiol 16:2869–2878.

Kohl K.D., M.M. Skopec, and M.D. Dearing. 2014*b*. Captivity results in disparate loss of gut microbial diversity in closely related hosts. Conserv Physiol 2:cou009.

Lessner K.M., M.D. Dearing, I. Izhaki, M. Samuni-Blank, Z. Arad, and W.H. Karasov. 2015. Small intestinal hydrolysis of plant glucosides: higher glucohydrolase activities in rodents than passerine birds. J Exp Biol 218:2666–2669.

Lindroth R.L. 1988. Hydrolysis of phenolic glycosides by midgut β-glucosidases in *Papilio glaucus* subspecies. Insect Biochem 18:789–792.

Lotan A. and I. Izhaki. 2013. Could abiotic environment shape fleshy fruit traits? a field study of the desert shrub *Ochradenus baccatus*. J Arid Environ 92:34–41.

Majak W. 1991. Metabolism and absorption of toxic glycosides by ruminants. J Range Manag 45:67–71.

McDonald J.H. 2014. Handbook of biological statistics. Sparky House, Baltimore, MD.

McFall-Ngai M., M.G. Hadfield, T.C.G. Bosch, H.V. Carey, T. Domazet-Loso, A.E. Douglas, N. Dubilier, et al. 2013. Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci USA 110:3229–3236.

McLean S. and A.J. Duncan. 2006. Pharmacological perspectives on the detoxification of plant secondary metabolites: implications for ingestive behavior of herbivores. J Chem Ecol 32:1213–1228.

McNeill L.K. and J.R. Hamilton. 1971. The effect of fasting on disaccharidase activity in the rat small intestine. Pediatrics 47:65–72.

Morant A.M., K. Jørgensen, C. Jørgensen, S.M. Paquette, R. Sánchez-Pérez, B.L. Møller, and S. Bak. 2008. β-glucosidases as detonators of plant chemical defense. Phytochemistry 69: 1795–1813.

Nakano H. and J.F. Gregory III. 1995. Pyridoxine and pyridoxine-5′-β-D-glucoside exert different effects on tissue B-6 vitamers but similar effects on β-glucosidase activity in rats. J Nutr 125: 2751–2762.

Németh K., G.W. Plumb, J.-G. Berrin, N. Juge, R. Jacob, H.Y. Naim, G. Williamson, D.M. Swallow, and P.A. Kroon. 2003. Deglycosylation by small intestinal epethelial cell β-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. Eur J Nutr 42:29–42.

Pankoke H., M.D. Bowers, and S. Dobler. 2010. Influence of iridoid glycoside containing host plants on midgut β-glucosidase activity in a polyphagous caterpillar, *Spilosoma virginica* Fabricius (Arctiidae). J Insect Physiol 56:1907–1912.

———. 2012. The interplay between toxin-releasing β-glucosidase and plant iridoid glycosides impairs larval development in a generalist caterpillar, *Grammia incorrupta* (Arctiidae). Insect Biochem Mol Biol 42:426–434.

Rawls J.F., M.A. Mahowald, R.E. Ley, and J.I. Gordon. 2006. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. Cell 127:423–433.

Samuni-Blank M., Z. Arad, M.D. Dearing, Y. Gerchman, W.H. Karasov, and I. Izhaki. 2013*a*. Friend or foe? disparate plant-animal interactions of two congeneric rodents. Evol Ecol 27: 1069–1080.

Samuni-Blank M., I. Izhaki, M.D. Dearing, Y. Gerchman, B. Trabelcy, A. Lotan, W.H. Karasov, and Z. Arad. 2012. Intraspecific directed deterrence by the mustard oil bomb in a desert plant. Curr Biol 22:1218–1220.

Samuni-Blank M., I. Izhaki, M.D. Dearing, W.H. Karasov, Y. Gerchman, K.D. Kohl, P. Lymberakis, P. Kurnath, and Z. Arad. 2013*b*. Physiological and behavioural effects of fruit toxins on seed-predating versus seed-dispersing rodents. J Exp Biol 216:3667–3673.

Samuni-Blank M., I. Izhaki, Y. Gerchman, M.D. Dearing, W.H. Karasov, B. Trabelcy, T.M. Edwards, and Z. Arad. 2014. Taste and physiological responses to glucosinolates: seed predator versus seed disperser. PLoS ONE 9:e112505.

Scharf M.E., E.S. Kovaleva, S. Jadhao, J.H. Campbell, G.W. Buchman, and D.G. Boucias. 2010. Functional and translational analyses of a beta-glucosidase gene (glycosyl hydrolase family 1) isolated from the gut of the lower termite *Reticulitermes flavipes*. Insect Biochem Mol Biol 40:611–620.

Silva M.C.P., W.R. Terra, and C. Ferreira. 2006. Absorption of toxic β-glucosidases produced by plants and their effect on tissue trehalases from insects. Comp Biochem Physiol B 143:367–373.

Struempf H.M., J.E. Schondube, and C. Martínez Del Rio. 1999. The cyanogenic glycoside amygdalin does not deter consumption of ripe fruit by cedar waxwings. Auk 116:749–758.

Torregrossa A.-M. and M.D. Dearing. 2009. Nutritional toxicology of mammals: regulated intake of plant secondary compounds. Funct Ecol 23:48–56.

Yapi D.Y.A., D. Gnakri, S.L. Niamke, and L.P. Kouame. 2009. Purification and biochemical characterization of a specific β-glucosidase from the digestive fluid of larvae of the palm weevil, *Rhynchophorus palmarum*. J Insect Sci 9:1–13.